EVALUATION OF SERUM CYTOKERATIN 18M30 LEVELS INCASES OF NON ALCOHOLIC FATTY LIVER DISEASE

Dissertation submitted for

M.D. BIOCHEMISTRY BRANCH – XIII

DEGREE EXAMINATION

THE TAMILNADU DR.M.G.R.MEDICAL UNIVERSITY

CHENNAI – 600 032

TAMILNADU

APRIL 2017

BONAFIDE CERTIFICATE

This is to certify that this dissertation work entitled **"EVALUATION OF SERUM CYTOKERATIN 18M30 LEVELS IN CASES OF NON ALCOHOLIC FATTY LIVER DISEASE"** is the original bonafide work done by **DR.J.VINODHA**, Post Graduate Student, Institute of Biochemistry, Madras MedicalCollege, Chennai under our direct supervision and guidance.

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Place: Chennai

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SPECIAL ACKNOWLEDGEMENT

The author gratefully acknowledges and sincerely thanks Professor **Dr.M.K.MURALIDHARAN, M.S., M.Ch (Neuro).,** Dean, Madras Medical College and Rajiv Gandhi Government General Hospital, Chennai, for granting his permission to utilize the facilities of this Institution for the study.

ACKNOWLEDGEMENT

The author expresses her heartfelt gratitude to her guide,**Dr.R.CHITRAA, M.D**., Professor, Institute of Biochemistry, Madras Medical College, Chennai, for her intellectual and valuable guidance, unfailing support, encouragement and continuous inspiration throughout the period of her study.

The author in particular, is extremely thankful to **Dr. MOHAMMED ALI,MD,DM**, Director and Professor ,Department of Gastroenterology and **Dr. SRINIVASAGALU** MD, Director and Professor ,Department of Internal Medicine, Rajiv Gandhi Government General Hospital, Chennai, for granting permission to obtain blood samples from the patients. The author is indebted to **DR.G.SHEILA RANI MD ,DGO**Medical Superintendant for the clinicaldata collected.

The author expresses her thanks to the **Director Dr.K.Ramadevi, and other Professors Dr.I.Periyandavar M.D**,**Dr** .**V.Amudhavalli**, **Dr.K.PramilaM.D andDr.Sumathy.S. M.D**Institute of biochemistry, Madras Medical College, for their guidance, encouragement, insightful comments and suggestions.

The author expresses her warm respects and sincere thanks to her co-guide. **Dr.V.Ananthan. M.D** Assistant Professor, Institute of biochemistry, Madras Medical College for his guidance and support. The author expresses her warm respects and sincere thanks to other **Assistant Professors, Dr.Karpagavalli.V.C**, **Dr.Mythili.,Dr.S.Siva, Dr.B.SudhaPresanna,Dr.Veena Juliette and**

Dr.MenakaShanthi Institute of biochemistry, Madras Medical College, for their valuable suggestions regarding the practical issues of research.

The author expresses warm respects to the members of the Institutional Ethical committee for approving the study.

The author expresses her special thanks to Biochemistry Laboratory Staff , for their timely help and cooperation during sample collection.

The author is indebted to the patients from whom blood samples were collected for conducting the study.

The author expresses her special thanks to her co-PGs Dr.PreethiAnandan, and Dr.V.Priyadarshini for their cooperation and genuine support. The author expresses her thanks to all her colleagues in the institute, for their constant encouragement throughout the study period.

The author is grateful to acknowledge the help rendered by Dr.Jegannathan for the statistical analysis of the study.

The author expresses her special thanks to her husband Mr.Gnana Moses, her mother Mrs. Alice, her father Mr.C.Jegannathan,daughter Sharon and her son Joshua and other family members for the moral support and encouragement extended by them which gave fulfilment to the dissertation work.

Above all, the author is grateful to the Almighty for providing this opportunity, without whose grace nothing could be accomplished.

CONTENTS

ABBREVIATIONS

1.	NAFLD	-Non alcoholic fatty liver disease
2.	NASH	-Non alcoholicsteatohepatitis
3.	GWAS	-Genome wide association studies
4.	PNPLA3	-Patatin-like phospholipase domain-containing 3 gene
5.	FDFT	-Farnesyldiphosphatefarnesyltransferase
6.	HGNC	-Hugo Gene NomenclatureCommittee
7.	COL131A1	-Collagen, type XIII, alpha 1
8.	PGDFA	-Platelet-derived growth factor alpha polypeptide
9.	LTBP3	-Latent transforming growth factor beta-binding protein 3
10.	NCAN	-Neurocan
11.	PPP1R3B	-Protein phosphatase 1, regulatory subunit 3B
12.	GCKR	-Glucokinase regulator region
13.	LYPLAL1 gene	-Lysphospholipaselike 1 gene
14.	PPAR	- Peroxisome proliferators activated receptors
15.	SREBP	- Sterol regulatory element binding protein
16.	CREBP	- Carbohydrate response element binding protein
17.	VLDL	-Very low density lipoprotein
18.	LDL	- Low density lipoprotein
19.	HDL	- High density lipoprotein
20.	DGAT	-Diacylglycerol acyl transferase
21.	PUFA	- Polyunsaturated fatty acid
22.	4-HNE	-Hydroxynonenal
23.	MDA	- Malondialdehyde
24.	HETE	- Hydroxyeicosatetraenoicacid
25.	ADRP	- Adipose differentiation related protein
26.	MTTP	- Microsomal triglyceride transfer protein
27.	AAP	- Aminoantipyrine

28. IDMS - Isotope dilution mass spectrometry
29. SdLDL -Small dense low density lipoprotein
30. LXR - Liver X receptor
31. GK -Glycerolkinase
32. PVS - Poly vinyl sulphonic acid
33. PEGME - Polyethylene-glycol-methyl ether
34. CHER - Cholesterol esterase
35. MES -Morpholinoethanesulphonic acid
36. IDOL - Inducible degrader of LDL receptor.
37. ROS - Reactive oxygen species
38. TRAIL - TNF related apoptosis – inducing ligand receptor
39. SOCS - Suppressors of cytokine signaling
40. TUNEL - Terminal deoxynucleotidyltransferase (Tdt) – mediated UTP nick – end labeling technique.
41. UPR - Unfolded protein response
42. DAMPS - Damage associated molecular patterns
43. PAMPS - Pathogen associated molecular patterns
44. TLR - Toll like receptor
45. LPS - Lipopolysaccharide
46. HFCS - High fructose corn syrup
47. MMPs - Matrix metalloproteinases

Introduction

INTRODUCTION

Non alcoholic fatty liver disease is a commonly occurring chronic liver disease which includes a spectrum of conditions of varying severity characterized by increased deposition of fat in the liver caused by factors other than significant alcohol consumption[1].

It is the most common chronic liver disease occurring globally especially in the developed countries [2].

The prevalence of the disease is not known exactly, but 9 – 37% of the general population is affected by the disease[3,4]. .
The prevalence and incidence is expected to rise, as the global obesity incidence grows in developing countries, with the trend as seen in western lifestyle [5,6].

The disease may affect any age group with the highest rate of incidence in the 40 to 49 years. It is the most common disease affecting the liver in the pediatric age group. It affects children mainly between ages 2 to 19[7].

It comprisesa broadspectrum of pathological conditions involving the liver with varying clinical prognoses. The clinically mild end of the spectrum, is the simple accumulation of triglycerides inside the hepatocytes (simple fatty liver or steatosis)[11].It progresses to the more severe non

alcoholicsteatohepatitis (NASH). In 10% of cases it may progress to cirrhosis in the next 10 years [12].

NAFLD includes the following stages,

1. Hepatocellular steatosis (simple fatty liver)
2. Steatohepatitis
3. Fibrosis
4. Cirrhosis

End stage liver failure or even hepatocellular carcinoma [13,14]constitute the most severe end of the spectrum.

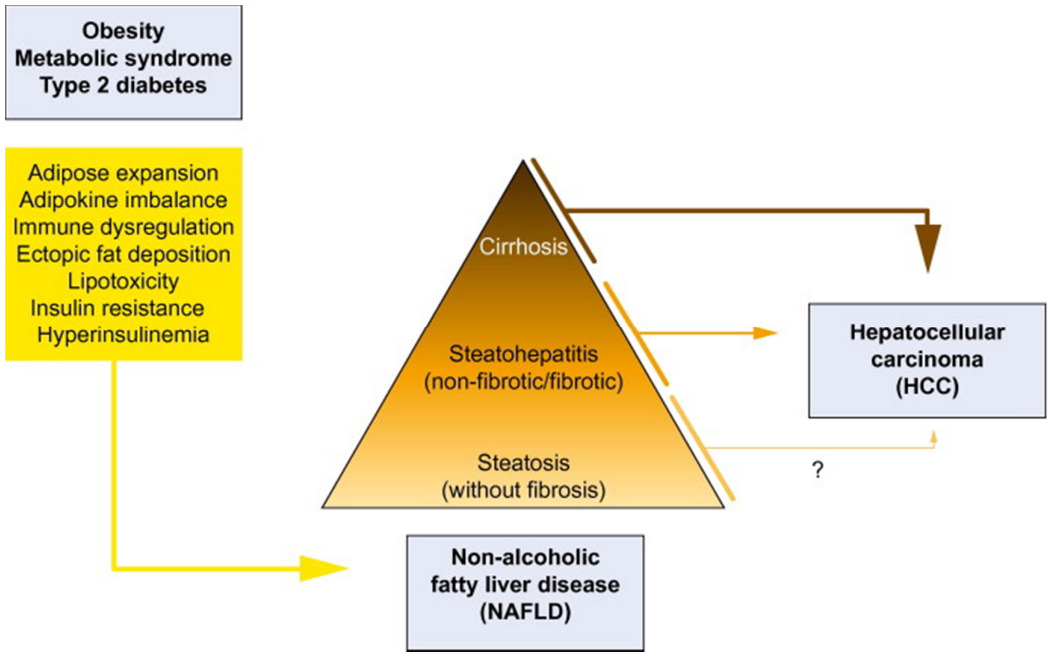


Figure 1: STAGES OF NAFLD

NAFLD is now recognized as an important component of metabolic syndrome and the main underlying pathophysiology of NAFLD, is **insulin resistance**. The metabolic syndrome constitutes a clustering of risk factors like atherogenicdyslipidaemia, hypertension [8,9], abdominal obesity [10] and elevated plasma glucose, that makes an individual more prone for developing atherosclerotic cardiovascular complications. There is now increasing evidence that NAFLD affects other organs too. It affects many extra-hepatic organs and regulatory pathways. For example, NAFLD increases the risk for chronic kidney disease (CKD)[11] too.

Liver transplantation is the only treatment for NASH related cirrhosis [12] and end stage liver failure.It has been proven that NASH related cirrhosis is the second leading cause for liver transplant next to hepatitis C virus related liver disease.With increasing industrialization and the global epidemic of NAFLD, cirrhosis caused by NASH is likely to become the leading cause of liver transplantation by 2030.

Abdominal imaging by ultrasonography[13], transient elastography (fibroscan) [14], computerized tomography and laboratory investigations comprising of abnormal liver enzymes cannot distinguish the subjects with non alcoholic fatty liver disease with steatohepatitis and subsequent fibrosis.

The gold standard for confirming the diagnosis, assessing the severity of fibrosis, grading the extent of liver damage and to follow up the response to

therapyin NAFLD is liver biopsy [15].The disadvantages of liver biopsy, are inconvenience to the patient, including pain and bleeding complications, sampling error and reducedperformance in obese patients. Sampling error is noted with all modes of liver biopsy.This is a tough procedure for the surgeon too needing expertise in the technique[16].

So a simple noninvasive test that accurately differentiates non alcoholic fatty liver from non alcoholicsteatohepatitis,as well as determines the severity of the disease would be of great clinical use.

Serum Cytokeratin 18M30 is a biomarker whose levels are progressively elevated in the various stages of non alcoholic fatty liver disease[17] .

When hepatocytes accumulate fat, they disrupt the intermediate filaments in the cytoplasm composed of cytokeratin. This results in the formation of Mallory bodies .A Mallory body is made up of of abnormally phosphorylated and cross linked keratins such as cytokeratin18.

The hepatocytes with Mallory bodies are more prone for apoptosis. So these keratins including cytokeratin 18 are released into the peripheral blood during apoptosis [18]. So, levels of cytokeratin 18 in the serum are progressively elevated in non alcoholic fatty liver (NAFL) and non alcoholicsteatohepatitis (NASH).

The aim of this study is to assess the serum levels of cytokeratin 18M30 in the various clinicalstages of non alcoholic fatty liver disease.

Early diagnosis coupled with therapeutic intervention, can control the progression of the disease and prevent the development of the worst complications which includes cirrhosis, liver failure and hepatocellular carcinoma[19].

Review of Literature

REVIEW OF LITERATURE

The liver is the largest organ in our body. The nutrientsthat are absorbed from the ingested food in the gastrointestinal tract as well as other substances including various drugs and bacterial metabolites are processed initially in the liver.Thus the liver processes useful metabolites but potentially harmful substances taken orally are detoxified in the liver[20]. Thereby, the liver functions like a gatekeeper.

The liver contributes a lot in maintaining the biochemical status of the body. The metabolic functions performed by the liver are numerous.It plays a major role in carbohydrate metabolism. It is one of the sites for gluconeogenesis, glycogenesis and glycogen storage [22]. Vital biochemical compounds are formed from intermediate products of carbohydrate metabolism.In the postprandial state the liver plays a crucial role in maintaining blood glucose levels within normal limits.It also helps in maintaining normal blood glucose levels over short (hours) and long (days to weeks) periods[22].

The liver plays a vital role in protein metabolism.The synthesis of glucose and lipids from the non nitrogenous parts of the amino acids takes place in the hepatocytes of the liver. Many of the enzymes used in these pathways including alanine aminotransferases and aspartate aminotransferases are measured to assess the severity of liver damage in acute and chronic diseases of the liver[23].

Lipid metabolism predominantly occurs in the liver.The majority of lipoproteins are synthesised in the liver.Excess of carbohydrates and proteins are converted into triglycerides and stored in the hepatocytes, which is the site for the synthesis of cholesterol and phospholipids.

Thus the liver is an important and altruistic organ [24],coordinating vital metabolic processes and maintaining biochemical status and homeostasis of the body.When there is a derangement in these metabolic pathways due to any cause, which may be environmental or genetic,there occurs an abnormal accumulation of the substrates and intermediates in the hepatocytes, altering their morphology and function.When it is a chronic cause, there is progressive damage to the hepatocytes, leading on to chronic liver disease which may even progress to end stage liver failure.

The commonest of such metabolic derangements isnon alcoholic fatty liver which occurs due to a variety of environmental and genetic factors[25].

It is one of the causes of fatty liver, which occurs when fat deposition in the hepatocytes, is more than 5 to 10% of the weight of the liver[26] when there is no history of excessive intake of alcohol.

The permissivelevel of alcohol intake varies but can be considered as alcohol consumption of not more than 20 grams/ day in males (21 drinks per week) and 10 grams / day (14 drinks per week) in females .

There has been a rapid increase in the incidence as well as prevalence of the disease and a dramatic rise in obesity and type 2 diabetes has been observed. The estimated prevalence ranges between 20 and 30% in the general population and increasing upto 90% in morbidly obese individuals. Non alcoholicsteatohepatitis (NASH), theclinically more severe form of the disease, is less common, and affects about 2–3% of the general population, and up to 74% of the morbidly obese[27].

NAFLD occurs in about 94% of obese individuals (BMI >30 kg/m^2), 67% of the individuals with overweight (BMI >25 kg/m^2), and 25% of thenormal individuals .

It is closely linkedto type 2 diabetes, and fatty liver occurs in 70% of type 2 diabetics screened with ultrasonography[28]. It represents the hepatic manifestation of the metabolic syndrome.

The prevalence of the disease in the pediatric population[29], is 3.2% and in obese children it is greatly increased to almost 53%[30].It occurs in all ethnic groups.

It includes a wide range of diseasepathology,ranging from simplesteatosis (simple deposition of fat in the hepatocytes, which is mostly stable) to non-alcoholic steatohepatitis (cellular ballooning, necro-apoptosis, inflammation and fibrosis, which is more severe) and progresses to cirrhosis[31].Cirrhosis is clinically the most severe end of the spectrum.

In a minority of cases it may worsen resulting in end stage liver failure or hepatocellular carcinoma (2 to 3%). Due to the rapid increase in prevalence,NAFLD related cirrhosis (cryptogenic cirrhosis) has become acommon indication for liver transplantation.

Historical perspective

Fatty liver was firstdescribed in the nineteenth century by the scientist Virchow. Heexplained the different classes of fatty liver.He also described the gross appearance of fatty liver including the yellowish discoloration,change in shape due to increased size and increased firmness of the liver.The globules of fat were proved to be inside the hepatocytes. The word “fatty liver” was coined by Ludwig[32].

Later on, in the 1870s Morgan explained a strong association between fatty liver and obesity and also with over-eating.

Several years later Zelman confirmed liver fibrosis and cirrhosis in obese patients without alcohol abuse. In 1970s, fatty liver was noted in patients who underwent intestinal bypass for treatment of morbid obesity.

Epidemiology

NAFLD is the commonestliver disease globally, with a worldwide prevalence of 6 – 33%. Whereas, in western countries it is about 20 -30%[33]**.**

In the Asian continent, the prevalence varies between 12 - 24 %[34].

The occurrence of NAFLD in Asian countries is much lesser than the western countries but, of late, ithas greatly increased in Asia too, due to the gradual increase in obesity, type 2 diabetes and metabolic syndrome in these regions.This is mainly due to the change in lifestyle and industrialization.

The prevalence is estimated to be around 5 - 28% in the Indian population[35].

Genetic basis

NAFLD is a complex disease trait in which minor inter-patient genetic variations act together with environmental factors to determine disease phenotype and its progression. These genetic variations that determinesteatosis, are being studied and mapped by GENOME WIDE ASSOCIATION STUDIES (GWAS)[36].

Several non-synonymous sequence variations were studied and a missense mutation [Ile148→Met148 (I148M)] was located in a PATATIN-like PHOSPHOLIPASE domain-containing, i.e., *PNPLA 3* gene *(PNPLA3)*(HCNG: 18590) [37].*PNPLA3*mutations are greatly expressed in adipose tissue and liver, and their actions are regulated by insulin through a signaling cascade which is greatly increased with feeds[38].

Additionally, the *PNPLA3* gene also,causes difference in the prevalence of fatty liver disease between various ethnic groups[39]. The prevalence of NAFLD is much higher in the Hispanics(Whites) when compared to the Black

Americans. Most probably, this may also be the reason for familial clustering of NAFLD. The frequency of *PNPLA3* gene was much higher in NAFLD patients, when compared to normal individuals,with a 3.3- fold increased risk[40].

The rs738409 single nuclear polymorphism, influenced both the presence of NASH and the severity of fibrosis in NAFLD patients, with histological evaluation of liver damage, independent of obesity and diabetes[41,42].

Five new genetic variants were found to be associated with progressive inflammation and fibrosis in NAFLD :

1. A variant in the *FDFT1* (farnesyldiphosphatefarnesyltransferase 1, HGNCI 3629) was correlated with NAFLD[43].
2. The collagen gene *COL13A1* (collagen, type XIII, alpha 1, HGNC 2190) was associated with lobular inflammation[43].
3. A single nucleotide polymorphism near the *PDGFA* gene (platelet-derivedgrowth factor alpha polypeptide, HGNC 8799);
4. The *LTBP3* (LATENT TRANSFORMING GROWTH FACTOR BETA-BINDING PROTEIN 3, HGNC 6716), and the
5. *EFCAB4B* (EF-hand calcium binding domain 4B, HGNC: 28657).

Genomewise association studies, identified four other SNPs which positively correlated with degree of steatosis and progression of NAFLD.

These were

1. neurocan (*NCAN*, SNP rs2228603),[44]
2. protein phosphatase 1, regulatory (inhibitor) subunit 3B *(PP1R3B, SNP* rs4240624).
3. glucokinase regulator (*GCKR,* SNP rs780094) and
4. lysphospholipase like 1 (*LYPLAL1*, SNP rs12137855).

*NCAN*regulates cell to cell adhesion and lipoprotein metabolism, and closely linked with biopsy provensteatosis.

GCKR is one of the regulatory factors in the metabolism of glucose.

LYPLAL1 coordinates the actions of*PNPLA3* protein, in the breakdown of the triglycerides.

Both *GCKR* and *LYPLAL1* were also proved to be closely linked with biopsy proven,lobular inflammation of the hepatocytes and fibrosis.

The SNP rs2645424 on chromosome 8, in the gene encoding FARNESYL DIPHOSPHATE FARNESYL TRANSFERASE 1 *(FDFT1)*, an enzyme involved cholesterolbiosynthesis, was found to be associated with progression of NAFLD.

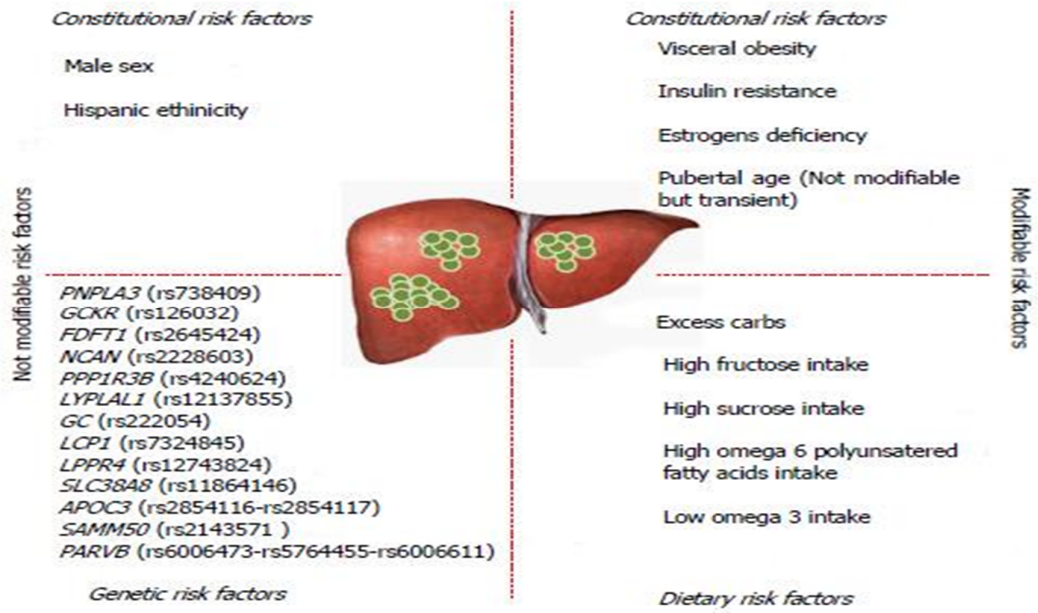


Figure2 :Genetic factors in NASH

Etiological conditions associated with non alcoholic fatty livermetabolic factors

- Metabolic syndrome:

1. Type 2 diabetes mellitus,associated with insulin resistance.
2. Obesity (especially truncal/central obesity).
3. Hyperlipidemia (especially hyper triglyceridemia).
4. Hypertension.
5. Polycystic ovarian disease.

Genetic / metabolic conditions

1. Lipodystrophies
2. Wilson’s disease
3. Abetalipoproteinemia
4. Galactosemia

5. Heriditary fructose intolerance

6. Systemic carnitine deficiency

7.Tyrosinemia

Toxin- associated steatohepatitis[(45)]

- Drugs

    1. Methotrexate

    2. Amiodarone

    3. Tamoxifen

    4. Nucleoside analogues

- **Occupation associated toxins**

    1. Carbon tetrachloride

    2. Ethyl bromide

    3. Petrochemicals

    4. Vinyl chloride

- **Malnutrition states and parenteral nutrition**

    1. Total parenteral nutrition

    2. Kwashiorkor

    3. Celiac disease.

- **Miscellaneous conditions**

    1.Inflammatory bowel disease

    2.Jejunal diverticulosis with bacterial overgrowth

    3. Gastric and jejunoileal bypass

Type 2 diabetes mellitus and insulin resistance in NAFLD

Insulin resistance is the core mechanism in NAFLD.

Hyperinsulinemia and insulin resistance can be noticed by the action of insulin on the target tissues like adipose tissue (continual lipolysis and reduced uptake of free fatty acids), muscle (diminished glucose release) and liver (reduced glycogenesis and glucose release).The development of diabetes mellitus is dependent on vitality of pancreatic islet cells but, it has been proved thatsteatosis mostly occurs, before development of diabetes in susceptible individuals.

Ethnicity significantly influences these associations. Previous studies prove that the fattyinfiltration of the liver occurs in three fourths of type 2 diabetes mellitus patients [(46)].Pathological changes in the liver worsen with deranged glucose metabolism and,diabetes mellitus in NAFLD doubles the risk for cirrhosis.

Obesity and NAFLD

Non alcoholic fatty liver is four to six times common in obese individualswhen compared to normal individuals by ultrasonography and abnormal liver enzymes[(47)].About 30% had significant fibrosis and 10% had cirrhosis.But ethnic factors significantly affect the relationship between obesity and NAFLD.

Metabolic syndrome and NAFLD

Obesity and steatosis, are major risk factors for metabolic syndrome having the common underlying mechanism of insulin resistance and lipotoxicity. There is an increased prevalence of about 60% of NAFLD in metabolic syndrome[48].

NASH in other liver diseases

Occult haemochromatosis and iron overload[49], is an important additional factor in the development of NASH. Moreover,hyperferritinemia is more closely associated with insulin resistance rather than iron overload and can be reversed by weight reduction.

Recently hepatic steatosis is also implicated as a major risk factor in the development of primary biliary cirrhosis.

Classification of NAFLD

Class 1:Simple fatty liver or simple steatosis (without inflammation or fibrosis)

Class 2:Steatosis with lobular inflammation without fibrosis or ballooned cell - Non alcoholicsteatohepatitis (NASH)

Class 3:Steatosis associated with inflammation/fibrosis of different degrees

Class 4:Steatosis associated with inflammation/fibrosis with ballooned cells containing MALLORY BODIES.

Class 1 and 2 are clinically mildconditions [50]

Pathogenesis

The pathogenesis of NAFLD can be described based on the “two hit hypothesis”.

The ‘first hit’, which has triglyceride deposition in the hepatocytes, causes steatosis .This makes the liver more prone to injury, caused by the following mediators so called “second hits” [51,52]

- Inflammatory Cytokines,
- Adipokines,
- Mitochondrial dysfunction
- Oxidative stress

These results, in cellular ballooning,steatohepatitis**,cell death**, fibrosis and cirrhosis.But free fatty acidstoo, have a direct role in promoting liver injury[53].

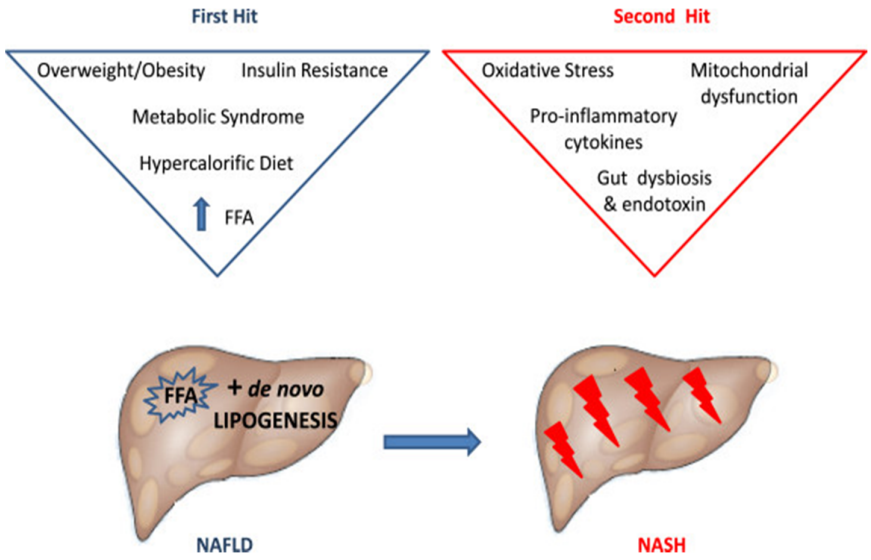


Figure 3:PATHOGENESIS OF NAFLD – THE TWO HIT HYPOTHESIS

Steatosis

Steatosis occurs when fat deposition in the liver is greater than 5 to 10% of the weight of the liver [54]. Excessive accumulation of fat in the liver parenchyma, occurs due to a derangement in the balance between **supply,synthesis, consumption and usage of triglycerides.**

The following are the sources of the lipids that contribute to the development of fatty liver

1. The uptake of non esterifiedfree fatty acids (NEFA) from the plasma which comes from lipolysis of the adipose tissue.
2. The newly synthesized fatty acids in the liver through de novo lipogenesis (DNL) from carbohydrate precursor.
3. Dietary fatty acids which reach the liver through uptake of intestinal chylomicrons.

Tracer studies done in NASH patients, who are obese,showed that 60% of triglycerides in the liver come from free fatty acids, 25% from *de novo* lipogenesis, and 15% from diet [55].

The disposal and usage of fatty acids proceeds in many ways;

1. esterification to triglycerides and incorporation into lipid dropletsin the cytoplasm.
2. lipoproteins such as VLDL, are formed and exported out of the liver.
3. oxidation of fatty acids through β oxidation in mitochondria.
4. synthesis of membrane phospholipids.

These pathways are regulated by energy homeostasis influenced by Peroxisome Proliferators Activated Receptors (PPAR) activity and the adrenergic nervous system.

The transcription factors consisting ofSterol Regulatory Element Binding Protein (SREBP)[56] regulated by insulin, and Carbohydrate Response Element Binding Protein(CREBP)[57], regulated by glucose levels play an important role in lipid metabolism. SREBP and CREBP,promote transcription of the enzymes regulating fatty acid synthesis, esterification into triglycerides in lipid droplets and thus promoting lipogenesis.

Fructose is a key player in NAFLD

Fructose play**s** a pivotal role in the pathogenesis of NAFLD[58].The composition of the food we eat, greatly influences deposition of hepatic fat. Carbohydrates,especially fructose,plays a key role in the development of steatosis. Increased fructose intake is strongly associated with adverse changes in lipid profiles.

High Fructose Corn Syrup (HFCS) has become a common constituent of our food items, from the 1970s. Soft drinks and sweetened beverages contain high quantities of fructose.They contain 55% fructose,41% glucose and 4% complex polysaccharides[60,61].

Fast food or “CAFETERIA” style food, are rich in high saturated fats, cholesterol and fructose. These fast food diet, promotes a gene expression that causes hepatic fibrosis, endoplasmic reticulum stress and lipoapoptosis.

The absorbed fructose from the intestinesis almost entirely extracted by the liver, amounting to 50 to 70% of the fructose reaching the liver[62].The anatomicallocation of the liver has gained an access to be in a key buffering position for absorption of amino acids and carbohydrates.Fructose extraction and metabolism, is significantly increased in the liver when compared to glucose, due to the extensive amount of fructokinasein the liver, which phosphorylates fructose.

The following are the mechanisms by which fructose induces hepatic steatosis.

- Increased fructose supply thrust stresson the hepatocytes, due to the activation of c-Jun-N terminal kinases and reduced hepatic insulin signaling leading on to insulin resistance[62,64].
- In fructose intake,dietary carbons are delivered to the liver in a state that is primed already to enter de novo lipogenesis.
- Fructose is phosphorylated at carbon 1, rather than carbon 6, which is converted to glyceraldehydes 3 phosphate which is the substratefor de novo lipogenesis.
- Fructose may prevent suppression of GHRELIN secretion[63] causing defectivesatiety mechanisms.

- When taken in large amounts,fructose can stress the liver by depleting hepatic energy supplies. After fructose injection,patients with NASH and normal individuals showed similar exhaustion of hepatic ATP levels, but the recovery of ATP levels to normal levels was much slower in NASH patients when compared to healthy subjects[63].

Previous studies which compared glucose and fructose metabolism in post-absorptive humans over short intervals have proved that fructose is metabolized much faster than glucose.

So by reducing fructose intake by avoiding fast food and soft drinks hepatic steatosis and NASH can be controlled.

Lipid composition (lipodomics) in NAFLD

Diacylglycerol Acyltransferase-1 (DGAT) is the enzyme responsible for esterification of NEFA into triglycerides in lipid droplets[65,66]. LIPODOMIC studies show an increasein the triacylglyceride to diacylglyceride ratio in NASH, compared to NAFL. So,triacylglycerides are elevated in severe NASH[67.68].

The ratio of n6:n3 polyunsaturated fattyacid (PUFA) is altered,[69.70] and the arachidonic acid levels are almost depleted in NASH. Free fatty acids were not different, but phosphatidylcholine was decreased in simple steatosis and NASH, while the free cholesterol to phosphatidylcholine ratio was increased. Ceramide, a toxic lipid intermediate, is found to be increased in

obesity associated NAFL, when compared with lean individuals presenting with fatty liver.

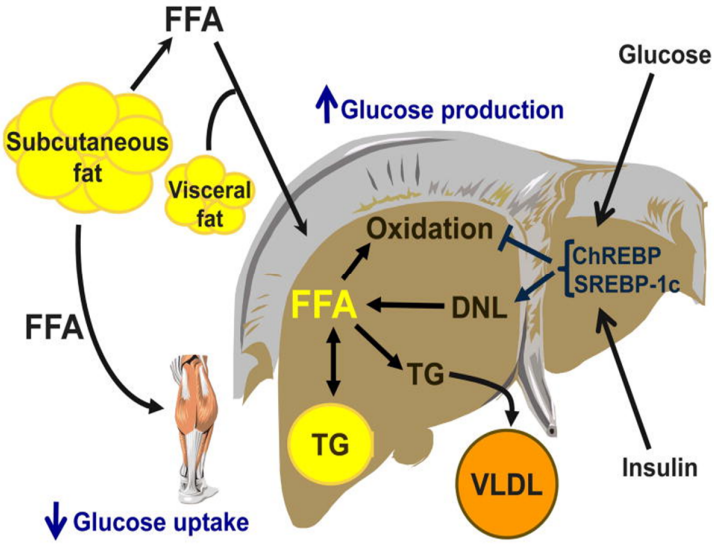


Figure 4 :LIPID HOMEOSTASIS IN NAFLD

Lipid peroxidation

Lipid peroxidation is the important feature that differentiates simple fatty liver from NASH.This is proved by the accumulation of oxidative by-products like malondialdehyde (MDA)[71],4-hydroxynonenal, nitrotyrosine, 8 hydroxydeoxyguanine, and systemic accumulation of adducts of oxidized phospholipids and 11 hydroxyeicosatetraenoic acid (11-HETE) [72] , which is a byproduct of arachidonic acid oxidative injury.

Lipid peroxidation occurs by branching chain reaction attack on unsaturated fatty acids that results in the production of a lipid hydroperoxide and another free radical.This is initiated mainly by superoxide radicals which are produced from mitochondrial oxidative phosphorylation in the electron

transport chain.Superoxide dismutase metabolises superoxide radical to hydrogen peroxide.This is converted to hydroxyl radicals (OH^-) in the presence of ferrous ions (Fe 2+) .This is called Fenton's or Haber-Weiss reaction. The hydroxyl radicals have a catalytic role in the peroxidation of fatty acids, which cause alteration and damage to cellular proteins, membranes and DNA.

Theoxidation of the phospholipid monolayer of lipid droplets composed of ,adipose differentiation related protein (ADRP), PERILIPIN AND TAIL interacting protein (collectively called PAT proteins) and constituent proteins of the endoplasmic reticulum are associated with cellular ballooning of hepatocytes,defective disposal of fatty acids and hepatic insulin resistance.The velocity of these biochemical reactions in the body, whether indays, hours , minutes or seconds or longer is not known.

VLDL lipidation andlipophagy

The fat droplets aresynthesized at the site of fatty acid acyl transferases in the endoplasmic reticulum in association with the cytoskeleton.Lipid droplets are associated with lipoprotein like proteins called PAT proteins which regulate lipase activity.These PAT proteins and are regulated by PPARγagonists.The growth of these proteins occurs principally by fusion.The triglycerides required for VLDL synthesis comes from the lipid droplets and this is activated by MTTP enzyme at the endoplasmic reticulum.Since PATprotein expression is altered by oxidative injury to the droplet surface ,the secretion and fusion of these droplets are disturbed in NASH .

Autodigestionof lipid droplets (lipophagy) occurs by formation of autophagosome (fusion with lysosome) is an important compensatory mechanism. This leads to alterations in the permeability of the autophagosome which is mediated by free fatty acids and triggers apoptosis and cell death.

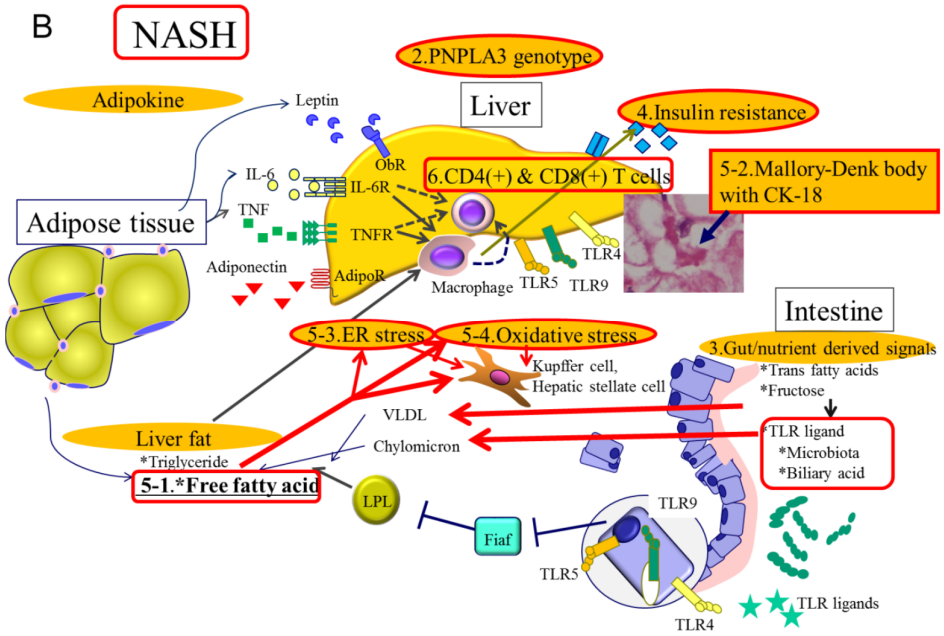


Figure 5 MOLECULAR MECHANISMS AND INFLAMMATORY CYTOKINES IN NASH

Endoplasmic reticulum dysfunction

Accumulation of lipid droplets results in formation of "Apo – B crescents" (rich in cholesterol) at sites in the endoplasmic reticulum[(73)].This causes stress and altered function of endoplasmic reticulum.This endoplasmic reticulum stress activates inflammatory mediators like nuclear factor κB (NF-Kb), interleukin 8 and c-jun N- terminal kinase (JNK) .So defective interaction of Apo B100 with lipid droplets at the endoplasmic reticulum produces an

association of oxidative stress with the endoplasmic stress reaction, lipid droplet accumulation,defective VLDL secretion and activation of pro-inflammatory pathways.

Dysfunction of mitochondria and ATP homeostasis

Steatosisis stronglyassociated with reduced ATP levels . This was first describedabout 50 years ago . P31 magnetic resonance spectroscopic studies have proved this recently .The history of mitochondrial evolution has placed it centrally in key pathways of synthesis of fatty acids and oxidation and apoptotic signaling.

Morphological changes in the mitochondria in NASH are

1. enlargement of the mitochondria
2. development of mitochondrial paracrystalline inclusions[74,75] which represent phospholipid phase transition.

This is due to defective electron transport chain[76] together with uncoupling of protein. The products of lipid peroxidation cause activation of mitochondrial UNCOUPLING PROTEIN (UCP – 2).UCP – 2 causes reduction in reactive oxygen species (ROS) by reducing ATP synthesis in the mitochondria.This results in ATP depletion under stress[77].

The increase in cholesterol content in mitochondria causesaltered mitochondrial permeability.This results in release of cytochrome C and triggers apoptosis[78].

Apoptotic cell death causing fibrosis

Apoptosis is programmed cell death that occurs by highly organized cellular and nuclear fragmentation.

During this process, cells are split up into fragments of small membrane-bound apoptotic bodies. These are digested and removed by phagocytosis.

Apoptosis can occur by two main pathways [(79)]:

- extrinsic pathway - mediated by death receptors present on the surface of the cell
- the intrinsic pathway - is organelle based.

The extrinsic pathway is initiated by apoptotic death receptors which includes Fas, TNF-R1 and TNF related apoptosis inducing ligand receptors(TRAIL).When activated, these receptors activate intracellular cascade systems which in turn stimulate proteolytic enzymes, mainly the caspases and cause cell death.

In the intrinsic pathway, many intracellular organelles stimulate apoptosis.Nuclear DNA damage,altered lysosomal permeability, endoplasmic reticulum stress, and mitochondrial dysfunction can trigger apoptosis.

Lysosomes release cathepsinB , a protease during TNF-α signaling , which causes mitochondrial dysfunction and cytochrome C is released[(80,81)].

Increase in TRAIL content in the cytoplasm increases lysosomal permeability and release of cathepsin B into the cytoplasm from the lysosomes[82,83].

In hepatocytes, mitochondrial dysfunction plays a crucial role by stimulatingthe apoptotic pathways and both the pathways are integrated into a common one .

Mitochondrial dysfunction releases many pro-apoptotic factors into the cytoplasm .This includes cytochrome c, which together with apoptotic-protein activation factor-1 (Apaf-1) and caspase 9, forms an activation complex, viz., the apoptosome.

This activation complex stimulates the effector caspases 3, 6 and 7 which complete the process of apoptosis and cause the final changes[84]. The Bcl-2 family of proteins consisting of pro-apoptotic (Bax,Bak) and anti-apoptotic (Bcl-2, Bcl-x_L)[85,86] regulate the mitochondrial actions of apoptosis.

Matrix metalloproteinase (MMPS) are also secreted by the activated HSCS which also promote hepatic fibrogenesis and TNF- α is released into the peripheral circulation. Animals experiments done in mice showed that diminished action of MMPS decreased liver injury, magnitude of apoptosis and fibrosis.

Lipotoxicity stimulates apoptosis

Numerous studies have proved that free cholesterol and saturated fatty acids(SFAS) which are critical in causing lipotoxocity also induce apoptotic signaling . Fatty acid transport proteins (FATPs) passively transport the FFAS across the hepatocyte membrane. Bechman et al explained that “FAT CD 36 / Fatty acid translocase was increased in NASH together with serum FFA and mediators of apoptosis. ”

TNF- α is an important inflammatory cytokine that is proapoptotic. Mari and his colleagues demonstrated that, excess free cholesterol made the hepotocytes sensitive to TNF-α, with subsequent apoptosis and progression to steatohepatitis.

Experimental studies prove that a cholesterol rich diet caused TNF- α stimulated cytochrome c release. Mitochondrial glutathione was also reduced.

TRAIL also promotes apoptosis. Malhi et al explained that FFAS made hepotocyies sensitive to TRAIL mediated apoptosis.

A combination of apoptotic signaling and focal necrosis results in necroapoptosis , which causes cell death.

The various responses to cell death include

- Cell repair,
- Inflammation,
- Regeneration,
- Fibrosis[87]

Among these responses, hepatic fibrosis is potentially the most deleterious[88], since progressive fibrosis may cause cirrhosis associated with subsequent grave complications of portal hypertension, liver failure and hepatocellular carcinoma.

Experiments in animalsand human studies have proved that, there is a link between hepatocyte apoptosis and liver fibrogenesis[89]. In animal studies,the reductionin the magnitude of hepatocyte apoptosis also produces reduction infibrogenesis[90,91].

The hepatic stellate cells(HSC) engulf the apoptotic bodies whichstimulates the fibrogenic potential of these cells[92].So the amount of hepatocyte apoptosis correlates with increasing magnitudeof fibrosis . Recent studies also prove that the nuclear DNA released from apoptotic cells also stimulate HSC cells and subsequent fibrogenesis[93].

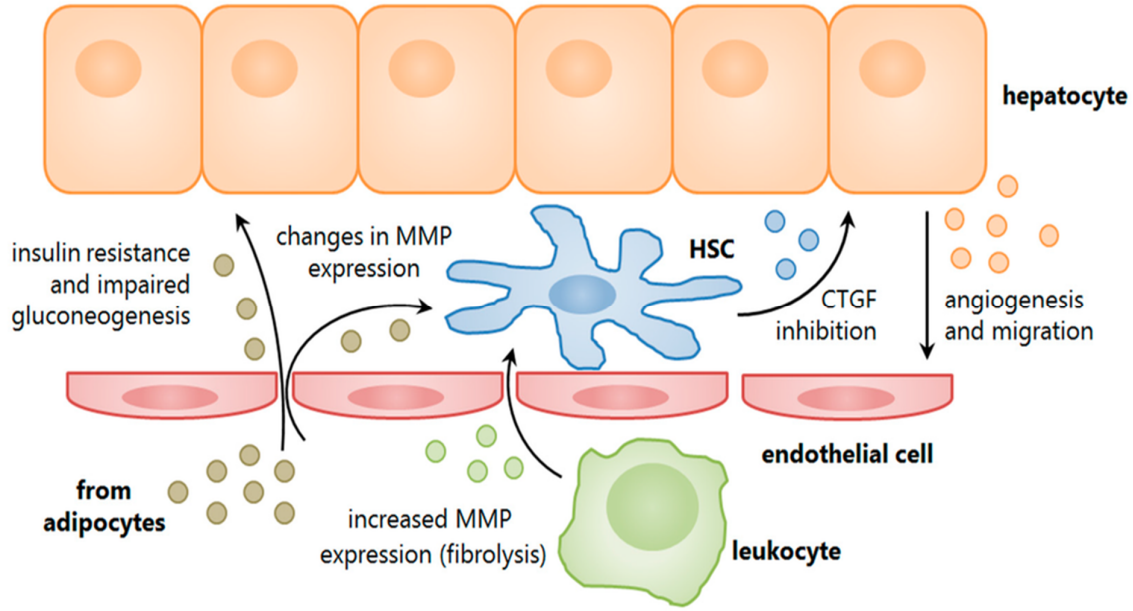


Figure 6 :FIBROGENESIS IN NAFLD

In NAFLD the mechanism of apoptosis in hepatocytes is as follows:

Excessive free fatty acids (due to reduced fatty acid binding protein)

↓

Alters autophagosome permeability

↓

Facilitates cathepsinB (lysosomal proteases) release

↓

Changes in permeability of mitochondria

↓

Release of cytochrome C andactivation of pro-apoptotic caspases (caspases 3,6 and 7)

Caspase 3 particularly causes disintegration of keratin 18 present in the intermediate filaments resulting in the formation of Mallory –Denk bodies and release of fragments of keratin 18 into the blood[94].

Previous studies using the TUNEL assay (terminal deoxynucleotidyltransferase-Tdt mediated dUTP nick end labelling technique) and immunohistochemical detection of caspase 3 in a large group of NAFLD patients, confirmed that hepatocyte apoptosis is the key pathological characteristic of human NAFLD[95].

The amountof hepatocyte apoptosis correlates with severity of fibrosis, AST/ALT ratio and degree of inflammation.

Apoptotic pathways are diffusely activated due to depletion of ATP , accumulation of lipid droplets and injury to the cytoskeleton, which contribute to hepatocyte ballooning.

Ballooned cells

Ballooning of the hepatocytes is the characteristic histological finding in simple steatosis and NASH [96,97].This occurs due to accumulation of fat, pushing the nucleus to the periphery.Ballooning is increase in cell size about 1.5 to 2 times the normal size on biopsy sections (normal diameter is 30 μm) with rarified cytoplasm[98].

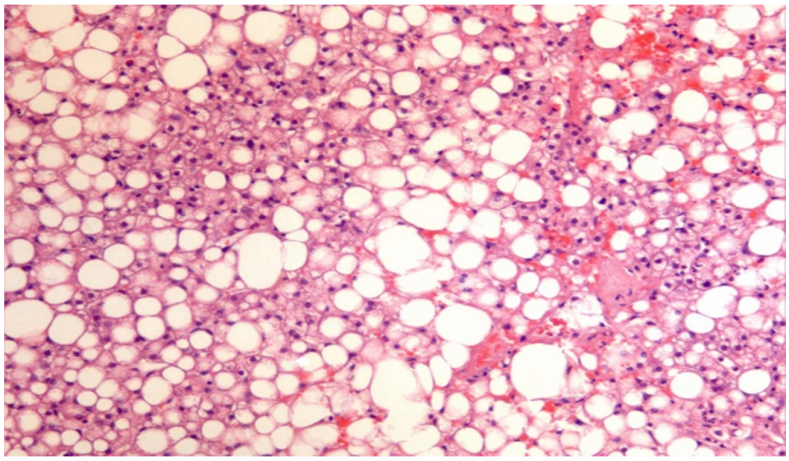


Figure 7: BALLOONED AND LIPID LADEN CELLS IN NAFL ON HISTOPATHOLOGICAL EXAMINATION

Ballooning due to lipid accumulation injures the cytoskeleton and intermediate filaments, forming Mallory-Denk bodies . Rupture of intermediate filaments releases fragments of cytokeratin 18 into the peripheral blood[99,100]. Ballooning of hepatocytes correlates with increasing fibrosis.

Insulin resistance causing inflammatory changes

Insulin resistance is the main hallmark of NAFLD, whichoccurs in most of NAFLD patients, and is responsible for development of the disease .

In normal subjects, insulin binding to its receptor phosphorylates insulin receptor substrates (IRS)-1, 2, 3 and 4, which results in propagation of the insulin signal.Phosphorylation of IRS-1 and 2 activates intracellular PI3K (phosphoinositide 3-kinase) and AKT/PKB (protein kinase B) pathways.These reactions are mainly responsible for the metabolic actions of insulin.The stimulation of AKT/PKB, translocatevesicles containing (glucose transporter 4) GLUT4 to the plasma membrane.Thispromotes glucose uptake[101].

Moreover theupregulation of forkhead box containing protein O (FOXO) transcription factor activity, facilitates the expression of important lipogenic genes with an associated reduction in glucogenic gene expression[102].

In insulin resistance,phosphorylation in the IRS changes from tyrosine phosphorylation to serine phosphorylation.resulting in reduced insulin action in the reactions mediated by phosphatidylinositol 3-kinase[103], AKT/PKB pathway and mitogenic pathways (mediated by Ras and mitogen activated protein).Inflammatory changes occur in theadipose tissue, due to endoplasmic reticulum stress and stimulation of Jun amino-terminal kinases (JNK).This causes the release of numerouscytokines, which changes tyrosine phosphorylationto serine phosphorylation.

Most of the biochemical abnormalities in NAFLD are due to interference with insulin signaling which results in insulin resistance. The factors which interfere with insulin signaling are free fatty acids, nuclear factor kappa B (NF-kB), ceramide, Jun N-terminal kinase 1 (JNK1), suppressors of cytokine signaling (SOCS) and cytochrome CYP2E.

Accumulation of intermediates of lipid metabolism likediacylglycerol (DAG) also reduces insulin signaling by protein kinase C (PKC) mediated mechanism. The above processes are also seen in skeletal muscles, which lead on to a systemicstate of insulin resistance[105,106].

Inflammatory cytokines in nonalcoholic steatohepatitis

Cytokines have a key role in the pathogenesis of NAFLD by facilitating hepatic inflammation, apoptosis and fibrogenesis.

TNF-α, an inflammatory mediator, is secreted by hepatocytes and Kupffer cells Previous studies have proved that,thereis a correlation between TNF-α expression and insulin resistance in NASH. Adipose tissue is a predominant site of obesity – mediated inflammation, facilitated by TNF-α expression, which causes inflammatory changes and insulin resistance. Elevated TNF-α levels were noted in obeseindividuals compared to lean individuals, and were positively correlated to insulin resistance[(106)].Circulating TNF-α levels was found to rise with increasing degrees of fibrosis in NASH.

In the liver, transforming growth factor β1 (TGF-β1) is secreted by stellate cells, Kupffer cells and epithelial cells. TGF-β1 facilitates hepatic fibrogenesis[(107)]by:

- stimulating stellate cells causing release of extracellular matrix proteins.
- transformation of resting stellate cells to myofibroblasts.

Polymorphisms involving TGF-β1 are linked to severe hepatic fibrosis in NAFLD patients with obesity.

Lipid homeostasis in NAFLD

Dysregulation of fat metabolism in NAFLD is characterized by increased production of very-lowdensity lipoproteins (VLDL), the key lipoprotein of the metabolic syndrome. Insulin resistance increases VLDL secretion in order to maintain hepatic lipid homeostasis. The small, dense LDL (sdLDL) which is highly atherogenic, is increased in NAFLD.

NAFLD associated dyslipidemic profile is characterized by:

Increased plasma VLDL,

Increased plasma LDL, and

Decreased HDL .

Many studies have documented the association between fatty liver and small dense LDL (sdLDL) concentration .The sdLDL are formed after removal of triglycerides from LDL. Since there is accelerated production and release triglyceride-rich VLDL into the plasma ,sd LDL, also get accumulated more.

These actions are facilitated by two enzymes.

First, cholesteryl ester transfer protein (CETP) promotes the triglyceride transfer from VLDL to LDL and cholesteryl esters from LDL to VLDL.

Secondly, hepatic lipase promotes lipolysis of triglyceride-rich LDL forming sdLDL.

So, CETP remodels VLDL in circulation, increasing its cholesterol content and also in conjuction with hepatic lipase, facilitates the sdLDL production[108].The mechanism of CETP is greatly increased in NAFLD .

LDL receptor exhibits a lesser attraction for smaller particles and so such sdLDL remain in the circulation for a long time.

Hyperlipidemia can also be caused by increased levels of apolipoprotein C-III (APOC-III), which inhibits lipoprotein lipase. APOC-IIIpolymorphismsalso cause NAFLD in humans.

Compared to simple steatosis, in steatohepatitis there is dysfunctional VLDL production and release.Fujita et al.compared serum lipoprotein levels between simple steatosis and NASH. Lipid profiles were same in the two groups; but the production and secretion of VLDLwere defective in NASH[109].

The defective hepatic VLDL export causes triglycerides to accumulate in the liver.MTTP is required for VLDL synthesis in the liver . MTTP polymorphisms causediminishedactivity of matrix metalloproteinases(MMPs) and secretion of VLDL which leads to greater triglyceride accumulation in the hepatocytes. This promotes development ofNASH . Also a fat rich diet was found to stimulate the methylation of MTTP and subsesequently lowers its mRNA level[110]. The postabsorptive phase also causes oxidative stress . Oxidized LDL stimulate HSCs that promote fibrogenesis in NASH.

Sortilins, intracellular sorting receptors for apolipoprotein B 100 (apo-B 100),attribute an important role in lipoprotein metabolism. Genome-wide association studies showed that sortilin 1 (*Sort 1*) gene was associated with metabolism of LDL. Numerous studies prove thatsortilin 1 is associated with hepatic metabolism oflipoproteins containing apo-B, but the exact mechanism is not clear.

Kjolby et al. confirmed that, “Sort 1 reacts with Apo-B 100 in the Golgi apparatus and promotesthe synthesis and export of VLDL lipoproteins, thus maintaining plasma LDL levels” [(111)]. They confirmed that over-expression ofSort 1promotesrelease of lipoproteins from the liver and elevated plasma LDL levels. Plasma LDL concentration, (the lipoprotein with highest cholesterol content in humans), is regulated by rate of synthesis and clearance by LDL receptors.

Fatty acid synthase (FAS) has a catalytic role in the final step ofde novo synthesis of fatty acidsand determines the maximal hepatic capacity to synthesize fatty acids. The expression of FAS mRNA in human liver is increased in hepatic steatosis.

Liver X receptors(LXRs) are nuclear receptors in hepatocytes that have a criticalrole in the lipid related genesis of NAFLD[(112,113)].

The various actions of LXR are:

- LXRs were found to serve as steroid sensors that regulate cholesterol metabolism[114,115]
- LXR stimulation promotes hepatic lipid accumulation and induces the synthesis as well as the export of VLDL particles from the liver.
- LXR stimulates SREBP-1c, the chief transcriptional regulator of de novo synthesis of fatty acid synthesis [116].
- LXR regulates insulin dependent stimulation of SREBP-1c .
- LXR can alsofacilitatelipogenesis by SREBP-1c independent mechanisms[117].
- LXRs can stimulate other transcriptional factors like ChREBP involved in lipogenesis[118].
- Theyinducethe CD-36 expression, fatty acid transporter, and scavenger receptor, which is another pathway by which LXR facilitates development of steatosis[119].
- They inhibit LDLR-mediated cholesterol uptake by stimulatingthe expression of inducible degrader of the LDLR (IDOL)[120]. IDOL promotes the ubiquitinization of LDLR anddegrades it.

Endotoxins and signals derived from the gut

Microbes in the gut have a role in NAFLD and NASH, HCC and atherosclerotic vascular disease, diabetes and other conditions.Endotoxins or lipopolysaccharides (LPS) secreted by the gut microbes may reach the liver

through the portal vein crossing the intestinal barrier. Patients with histological evidence of NAFLD have increased intestinal permeability with disrupted intercellular tight junctions in the intestine[121]. These changes are associated with bacterial overgrowth in the small intestine. The overgrowth of bacteria in the intestine cause complex changes and increased intestinal permeability with a concomitant decrease in the expression of tight junction proteins[122].

Diet rich in fat causes a rise in LPS levels two- to three times[123] . Pro-inflammatory cytokines induce inflammatory changes in the liver of NAFLD patients , but research has also proved that accelerated hepatic steatosis and inflammation occur by the influx of toxic metabolites into the portal vein in animal experiments, when inflammatory signals have been cut off [124] .

The gut and oral periodontal status have significant positive correlation with the onset and development of liver disease[125] . Treating periodontitis could cause improved transaminase levels in NAFLD and, in fact, starting on probiotics that reduce gut microbiota, improve NAFLD[126 ,127] . Previous studies using models of hepatocarcinogenesis have confirmed that a diet rich in fat causes increased levels of deoxycholic acid, a bacterial metabolite from the gut, damages DNA and accelerates hepatocarcinogenesis[128]. Microbes from thegut not only exacerbate NAFLD, but also obesity-linked hepatocarcino-genesis.

Toll-like Receptors (TLRs)

Toll-like receptors are sensitve to microbial and endogenous danger signals that are expressed in innate immune cells and in hepatocytes and they are linked to development and progression of NASH. Gut microbiota might produce pathogen- or damage-associated molecular patterns (PAMPs or DAMPs), which are TLR ligands that follow the activation of downstream signals. Ten TLRs (TLR1-10) have been recognized in humans and 13 (TLR1-9, 11–13) were detected in mammals. TLR2, TLR4, and TLR9 seem to have a role in the pathogenesis of NASH.

Toll-like receptor 2 is a receptor for numerous glycolipids or lipoproteins in bacteria adhering to the cell surface of monocytes or mast cells.Levels of free cholesterol are increased in hepatic stellate cells (HSC) in NAFLD resulting in elevated TLR4 protein levels and fibrogenic HSCs[(129)] . Kupffer cells are phagocytic cells that engulf different viral, or bacterial components and secrete hepatic pro-inflammatory and pro-fibrogenic cytokines. Cholesterol phagocytosed by Kupffer cells can activate these cytokines as well as increased expression of TLR4.

Clinical features of NAFLD

Symptoms and signs of NAFLD

Majority of NAFLD patients are without any symptoms. Most of them are diagnosed,when a liver imaging is done for other unrelated symptoms, i.e., when clinical assessment is done for metabolic syndrome.

When symptomatic, they present with, mostly nonspecific symptoms.

As in other chronic liver diseases, fatigue is the usual complaint. But, the severity of fatigue does not vary proportionately with the severity or the histological staging of the liver disease[130].

Few patients complain pain in the right upper quadrant of the abdomen (often positional) due to fatty infiltration and its stretching effect on the Glisson's capsule.Discomfort may be mistaken for gall stone disease.

Pruritus is less common with mild edema and on progression, anasarca occur only on development of cirrhosis.

On physical examination, obesity is the predominant clinical feature.

In other patients, the common clinical features of the metabolic syndrome,like hypertension is seen.

About 30 to 90% of the patients with NAFLD are obese and about half of them, present with mild hepatomegaly[131,132], which is usually due to steatosis and may also be due to glycogenesis, in diabetic patients.

Muscle wasting may occur with progression of the disease, but cannot be seen overtly due tocoexistent obesity in these patients.

Jaundice may be rarely seen in advanced stages of the disease.

Ascites, splenomegaly, spider angiomas, palmar erythema and caput medusae may be present in few patients with NASH-related cirrhosis [133].

AST and ALT in NAFLD

Mild to moderate increase in serum aminotransferase levels is commonly found in NAFLD patients(mean range 100 – 200 U/ L). Liver enzymes may be normal in a large percentage of patients, and normal levels of aminotransferases do not exclude presence of advanced disease.

Earlier studies have proved that the elevations in alanine aminotransferase(ALT) and aspartate aminotransferase (AST) are typically mild when present in NAFL and elevated two to three times the upper limit of normal in NASH patients. The AST/ALT ratiois usually < 1 in patients who have either absence or minimal fibrosis, and this ratio may be > 1 with the progression of fibrosis and development of cirrhosis [134].

Gamma-glutamyltransferase (GGT) levels are often increased. However, NAFLD cannot be diagnosed using GGT alone. Increasing levels of serum GGT are correlated with increasing severity of fibrosis in NAFLD patients.

Alkaline phosphatase may be slightly increased, and rarelyit may be the only abnormal liver function test.Albumin,bilirubin and platelets are usually normal and may be increased when cirrhosis develops.

Ferritin levels are increased in about half the patients with NASH , and transferrin saturation is increased in about 10%. Few NAFLD patients have low titres of autoimmune antibodies (anti nuclear and anti smooth muscle antibodies)

Ultrasound in NAFLD

In asymptomatic patients, ultrasound is a non-invasive and easily available tool for diagnosing pathological conditions of the abdomen.It is highly accurate in diagnosis, when sonographic criteria specific for NAFLD, are used in the diagnosis.Bright hepatic echoes, increased hepatorenal echogenicity and vascular blurring of portal or hepatic vein are the unique sonographic features of NAFLD.The typical findings on ultrasonography have been found to be the strongest independent predictor on multivariable analysis.

Diffuse steatosis (fatty liver)may be mild , moderate or severe based on the following findings.

Mild :Minimal diffuse increase in hepatic echogenecity , normal visualization ofdiaphragm and intra hepatic vessel borders.

Moderate :Moderate diffuse increase in hepatic echogenecity, slightly impaired visualization of intrahepatic vessels and diaphragm.

Severe :Marked increase in echogenecity, poor penetration of the posterior segment of the right lobe of the liver and poor (or) non visualization of the hepatic vessels and diaphragm.

The specificity and sensitivity of ultrasound was found to be about 88% to 95% and 60% to 94%.But, the sensitivity diminishes inmilder fatty infiltration. When there is ≥ 30% infiltration of fat, the sensitivity is 80% but it is 55% when hepatic fat content is 10% to 19% [(135)].

Moreover, the ultrasonography sensitivity for the detection of steatosis progressively decreases as the BMI increases, The sensitivity is, as low as 39% in individuals with BMI of 30 or more.

Another major drawback of ultrasound is the significant intra- and inter-observer variability. A previous study of 168 ultrasonographicevaluations for assessment of hepatic steatosis showed inter- and intra-observers' agreement of 47.0%-63.7% and 54.7%- 67.9%, respectively.

Transient Elastography (Fibroscan)

Transient elastography also called fibroscan , is based on measuring shear wave velocity.It measures the degree of liver stiffness and thus the degree of fibrosis in the liver.

The probe of the fibroscan device is placed in an intercostal space over the right lobe of the liver. A 50 MHz elastic shear wave is transmitted from a small transducer at the end of the ultrasound probe into the liver.There is also another transducer in the probe, which can detect the speed of the shear wave (in meters/ sec) as the wave is transmitted through the liver.The speed of the

shear wave is a measure of thestiffness of the liver, which is expressed in kilopascals (kpa).The whole process is known as liver ultrasound elastography.

Fibroscan can measure a liver volume of about 3 sq.cm which is 200 times > than liver biopsy.

Advantages of fibroscan

- Non- invasive
- Point of care testing
- No pain
- Sedation is not required
- Quick test and is completed in 5 to 7 minutes.
- No side effects

Disadvantages of fibroscan

It cannot be used in patients with

- Ascites
- Large amount of fat in the chest wall
- Morbid obesity[136]

Fibroscan can detect and quantify steatosis if the Controlled Attenuation Parameter (CAP) probe is used[137].It correlates with the decrease in amplitude of ultrasound waves as they propogate through the liver fat.It is based on the fact that fat affects ultrasound propogation. Hence, the more the steatosis, the higher, the CAP result will be.

Computerised tomography,transient elastography, magnetic resonance imaging and magnetic resonance spectroscopy are other newer modalities which can directly measure the quantity of hepatic fat.

Liver biopsy in NAFLD

Liver biopsy is currently the gold standard to make a definite and accurate diagnosis of the disease.

It is useful to differentiateNAFLD from other differential diagnosis,staging fibrosis, grading activity, for follow up , and assessing the response to treatment. But, liver biopsy is unreliable in early cases and has numerous disadvantages. They include :

1. Sampling error: Only about 1/50000 of the entire liver isexamined during a liver biopsy.So sampling error is a great problem. To avoid this, an adequate amount of tissue should be obtained. Collection of 2 or more samples from different representative areas will reduce interregional variation[(138).]
2. Inter and intra-observer variability–Error in staging fibrosis is minimized by obtaining a specimen > 2cm in length and 1.5mm diameter with a minimal length of 1.5 cm core needle using a 15-16 gauge needle.
3. Invasive procedure
4. Bleeding complications[(139)]
5. Patient risk

6. Pain and inconvenience to the patient
7. Performance in obese patients
8. Costly technique

Therefore, liver biopsy which is an invasive procedure is not practically suited for diagnosis of such a common condition. So a simple and noninvasive test has to be developed and validated to identify NAFLD.This test should be reproducible and reliable, that is both diagnostic and highly accurate to differentiate NASH from NAFL and for grading and staging of the disease.It should help to noninvasively monitor progression of the disease and response to treatment. Numerous researchers have attempted to develop a simple biomarker fordiagnosing NASH and Cytokeratin 18 M30is one of them

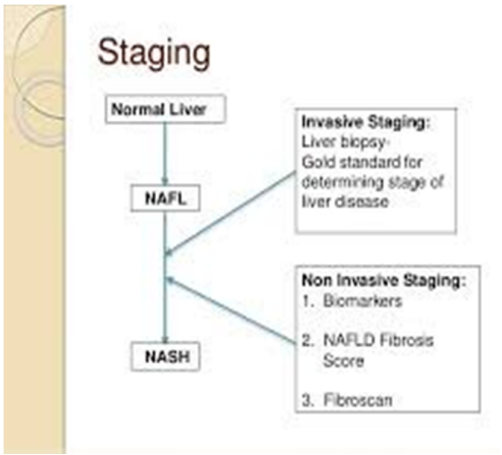


Figure 8 : Diagnostic Methods of NASH and NAFL

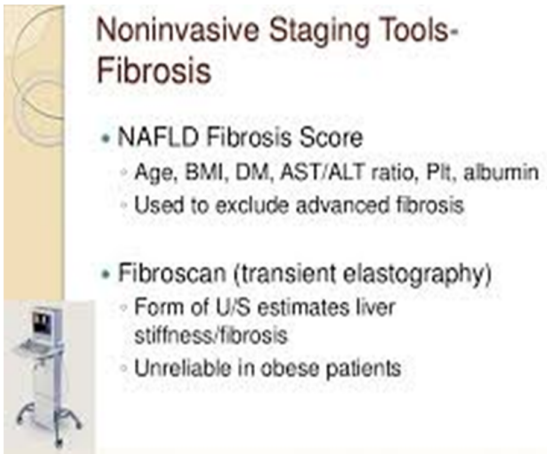


Figure 9:Non invasive diagnostic methods for NASH

Cytokeratin 18M30

Cytokeratins are a family of water insoluble, intracytoplasmic ,fibrous, structuralproteins that are the predominant components of intermediate filaments of epithelial cells[140,141].

The term cytokeratin was first used in the late 1970s by Schmide and Frank when the protein was first identified.

There are two types of cytokeratins:

1. the type I cytokeratins which are acidic and
2. thetype II cytokeratins which are basic or neutral.

They usually occur in pairs .Each pair consists of a type Iand a type II cytokeratin.

These proteins are obligate heteropolymers with equimolar concentrations of type I and type II proteins[142].

Cytokeratin 18, a type II protein is expressed on chromosome 12q18:13 About 25 subtypes have been classified based on molecular weight (from 48 to 68 kDa) and isoelectric pH (from 6 to 8).

CK18 couples with CK 8 in the intermediate filaments in the epithelial cells of the liver[143].CK 18 amounts to about 5 % of total amount of proteins in the hepatic cells and epithelial cells.

Within the cell, cytokeratin filaments form a dense complex network radiating from the nuclear surface to the cell surface.

They form a cytoplasmic scaffold that gives epithelial cells the ability to withstand mechanical as well as thenon mechanicalstress[144].

The link between the cell surface and the nuclear surface helps in the organization of the cytoplasm,providing tensile strength to the cell and also in cellular communication mechanisms. They interact with desmosomes and hemi-desmosomes to favor cell to cell adhesion and basal cell connective tissue interaction[145].

Apart from the relatively static functions performed by cytokeratin , cytokeratin intermediate filaments are highly dynamic structures which reorganize during mitosis and apoptosis. They are important for maintaining mitochondrial structures and for cellular processes like cell-cycle progression and cell signaling.

Reorganization is mediated by post translational phosphorylation, glycosylation, transglutamination and proteolysis or by interaction with other proteins.

The intermediate filaments of the cytoskeleton associate with the ankyrin and spectrin complex close to the cell membrane.

The dynamic equilibrium of thephosphoglycoprotein CK 18, in the soluble and filament pool, determines its cellular functions , and it is proved to be regulated by site -specific phosphorylation[146] .

In NAFLD, severity of disease is due to progressive apoptosis of the lipid accumulated cells in the liver.

Both the extrinsic and intrinsic pathways of apoptosis start at the mitochondria . Permeability of outer mitochondrial membrane promotes discharge of proteins from the inter mitochondrial space, into the cytoplasm.

This causes stimulation of pro-apoptoticcaspases including caspase 3,6 and 7 (mainly caspase 3) which cleaves many substrates inside the cell causing proteolysis of many cellular proteins including cytokeratin 18 in the intermediate filaments.During apoptosis, K18 fragments are dramatically reorganized and K18 is cleaved by caspases, which are cysteine proteases at multiple sequence sites[147].Cleavage at the C‑terminal domain 396 forms CK18–Asp396 neoepitope,known as M30 antigen . This neo-epitopeis formed when caspase cleavage of CK18 occurs at the position after the aspartic acid residue 396. Cleavage at this position is executed by caspase 9 in the early phase ofapoptosis and by caspase 3 and caspase 7 in the execution phase .

Cleavage of K18 is an early event occurring during apoptosis[148]. Fragmentation of cytokeratin 18 occurs and the fragments are released into the

extracellular space during cell death. So soluble cytokeratin 18 is one of the factors of hepatocyte apoptosis[149].

The M30 antibody recognizes this neo-epitope CK18-Asp396. Development of the M30 antibody into an ELISA had great clinical utility in measuring this specific apoptosis product in the blood.

The M30 ELISA measures the amounts of soluble CK18 fragments containing the CK18Asp396 neo-epitope. Caspase-cleaved K18 fragments are highly stable in human serum, further making it more convenient andeasy to analyze[150].

The measured levels of CK18M30 in serum, correlates well, with the degree of steatosis and the degree of fibrosis in NAFLD patients.Now, it can differentiatemoderate and severe fibrosis, especially in progressive cases of NAFL and NASH[149].

Serum uric acid levels in NASH

Uric acid (2, 6, and 8- Tri hydroxy purine) is a heterocyclic organic compound. Its molecular weight is 168 Daltons. It is a weak organic acid with a pKa of 5.75. At physiological pH, it significantly exists as urate which has higher solubility than uric acid. At a urine pH below 5.75, uric acid is the predominant form.

The amount of uric acid synthesized per day is about 400 mg and from dietary source is 300 mg.

In purine free diet, the total body pool of exchangeable urate is 1200mgs in men, and it is about 600mgs in women. It is the end product of purine metabolism.

Catabolism of purine nucleotides begins by removing ribose linked phosphate catalyzed by purine 5'-Nucleotidase. Removal of ribose moiety frominosine&guanosine by purine nucleoside phosphorylase produces Hypoxanthine and Guanine. Both are converted to xanthine, which is catabolised to uric acid by the action of xanthine oxidase.

Uric acid is degraded in mammalian creatures by enzyme uricase to produce allantoin, which is freely excreted in the urine.

Uric acid is higher in men as well as inpost menopausal women but, lower in females because, estrogens are uricosuric.

Many epidemiological studies have confirmed that uric acid is an independent risk factor for metabolic syndrome and cardiovascular disease[(151)]. In metabolic syndrome,an inverse correlation exists between insulin resistance and renal uric acid clearance which causes elevated serum uric acid levels[(152)].Increased triglycerides synthesis may increase uric acid production and excretion.

A Chinese study confirmed that, a positive association of elevated ALT and Met196Arg variant in $TNFRSF_1B$ with serum uric acid concentrations

among patients with NAFLD[153], supported this proposal called damage associated molecular patterns (DAMPS) and proved that release of uric acid may be accelerated when tissue injury combined with a genetic susceptibility happened in NAFLD.

Elevated serum uric acid is independently associated with NAFLD regardless of insulin resistance.

Normal reference range:

Male: -4.4to7.6 mg/dL,

Female: -2.3-6.6 mg/dL

Increased serum uric acid levels are seen in gout, excess dietary purine intake, increased nucleic acid turnover (Leukaemia, Chemotherapy, Radiotherapy, trauma) and preeclampsia.

Hyperuricemia is closely associated with insulin resistance, metabolic syndrome and cardiovascular conditions.

Uric acid has antioxidant properties in the extracellular environment, suggesting that it may have a protective role against disease.

But, multiple studies have shown that when uric acid enters cells via specific transporters it has a pro-inflammatory role. Inside the cell, uric acid can actas a pro-oxidant, causing the release of inflammatory mediators and growth factors.

Moreover, uric acid has been proved to contribute to lipoprotein oxidation andinflammation,which are important mechanisms in the development and progression of NAFLD. Preliminary evidence suggests that hypouricemic therapy can reduce the degree of hepatic steatosis in experimental studies.

Aims & objectives

AIM OF THE STUDY

1. To evaluate the levels of serum cytokeratin18M30 in the various stages of non alcoholic fatty liver disease – simple fatty liver (steatosis) and non alcoholicsteatohepatitis(NASH) diagnosed by ultrasound and transient elastography (fibroscan) and compare it with apparently healthy controls .

2. To correlate lipid profile (Total cholesterol,serum triglycerides, low density lipoprotein and high density lipoprotein) and serum uric acid with cytokeratin18M30 in various stages ofNAFLD.

Materials & methods

MATERIALS & METHODS

This is a cross sectional case control study and was conducted after getting ethical committee approval. The study comprised of a total number of 85 subjects including 35 patients of simple fatty liver and 25 patients of NASH diagnosed by ultrasonography in the Medical gastroenterology departments and Medical departments (outpatient and inpatients) and 25 normal controls in Rajiv Gandhi Government General Hospital, Chennai.

Inclusion criteria :

Cases of NAFLD including non alcoholic fatty liver (NAFL)and non alcoholicsteatohepatitis (NASH) diagnosed by ultrasonography and transient elastography (fibroscan).

Exclusion criteria:

- Patients with history of alcohol consumption (> 30 g /day for males and 20 g / day for females)
- Viral hepatitis
- Autoimmune liver diseases
- Hepatocellular carcinoma patients.
- Congenital liver diseases

Cases were selected based findings of fatty liver and fibrosis (steatohepatitis) in ultrasonography and transient elastography.

They were divided into two groups-

1. Non alcoholicfatty liver (NAFL)
2. Non alcoholic fatty liver (NASH).

Non alcoholic simple fatty liver was diagnosed based on the findings of fatty liver (steatosis) – grade 1, 2 or 3 by ultrasonography with normal findings on fibroscan (< 7 kpa).

NASH was diagnosed based on,ultrasonographicfindings of fatty liver and fibroscan values of > 7 kpa and elevated ALT and AST levels (twice or thrice normal) or AST/ALT ratio > 1.

In our Hepatology department fibroscan values are interpreted as follows

<7 kpa - Normal

7 to 9 kpa – Early fibrosis

9 to 13 kpa – Advanced fibrosis

>13 kpa - cirrhosis

A control group with normal ultrasonographic findings with normal fibroscan findings was also included in the study.

Blood sample collection:

- Blood was collected after 8-12 hrs of overnight fasting.

- About 5mL of venous blood was collected from antecubital vein after aseptic precautions and transferred in to 2 tubes and the investigations performed as per the following table.

Tubes	Anticoagulant	Amount of blood	Investigations
Tube1	---	3Ml	Serumcytokeratin18M30, Fasting Plasma Lipid profile,AST,ALT,ALP,Uric Acid.
Tube2	EDTA	3Ml	Fasting Plasma Glucose

The blood samples were analysedwith in 4 hrs of sample collection and were analysed by the following methodologies.

Estimation of serum cytokeratin 18 M30

Method:Quantitative sandwich enzyme immunoassay.

Kit from Cusabio

Principle.

- A monoclonal antibody specific for CK 18- M30 is already precoated on to a microplate.
- Samples and standards are pipetted into the microplate wells and the CK-18M30 present is bound by the immobilized antibody.
- After removal of any unbound substances ,a biotin- conjugated antibody specific for CK 18-M30 is pipette into the wells.

- The microplate wells are then subjected to washing ,avidin conjugated Horse Radish Peroxidase (HRP) is pipetted into the wells.
- Any unbound avidin-enzyme reagent is removed by further washing.
- Then a substrate solution is pipettedinto the wells and a color develops which is proportional to the amount of CK 18-M30 present in the samples.
- The colour development is stopped & intensity of colour is measured at 540 nm and 450 nm.The difference between the two readings is taken for correction of optical imperfection in the plate.
- Reagent composition:
    1. CK 18-M30 microplate -96 microwells coated with CK 18-M30 monoclonal antibody.
    2. CK 18-M30 standard (freeze dried) – 2Nos
    3. Biotin- antibody -120µL
    4. HRP - avidin- 120µL
    5. Wash buffer concentrate – 20 mL
    6. Sample diluent -50 mL
    7. Wash buffer concentrate – 20 Ml
    8. TMB substrate –10 mL
    9. Stop solution - 10 mL

Working reagent preparation:

- Biotin - antibody :- must be diluted 100 times . 10 µL of Biotin antibody added to 990 µL of Biotin antibody diluent.
- HRP - antibody :- 100 times dilution is needed . 10 µL of HRP antibody added to 990 µL of HRP antibody diluent.
- **Wash Bu**ffer Concentrate is diluted (x25) by diluting 20 mL into 480 mL of deionised water 500 mL of wash buffer is prepared.

PREPARATION OF STANDARD

- The freeze dried standard is mixed with 1.0 mL of sample diluent. Allow it to sit for 15 minutes with gentle agitation beforemaking serialdilutions.
- 250 µL of Sample diluent is pipetted into 7 tubes (S_0 to S_6).
- Using the stock solution a two- fold dilution series is prepared .Each tube is mixed thoroughly before the next transfer.
- The undiluted standard acts as the high standard (1000 m IU /mL).
- Sample diluent is the zero standard (0 m IU / mL).

Tube	**S7**	**S6**	**S5**	**S4**	**S3**	**S2**	**S1**	**S0**
mIU/mL	1000	500	250	125	62.5	31.2	15.6	0

PROCEDURE

- All reagents and working standards are prepared.
- 100 µL of standard and sample is added in each well.
- The plate is covered with adhesive strip and incubated at 37℃ for 2 hours.
- Without washing the liquid of each well is removed.
- 100µL of biotin antibody is added to each well.
- Plate is covered and incubated at 37°C for 1 hour.
- After aspirating each well, the wells are washed, repeating the process twice for a total of three washes.
- 100µL of HRP – avidin is added to each well .
- Plate is covered and incubated at 37℃ for 1 hour.
- The aspiration / wash process is repeated five times.
- 90 µL of TMB subsrate is added to each well.
- Plate is covered and incubated for 15 – 30 minutes at 37° C.
- 50 µL of Stop solution is added to each well and gently tapped for thorough mixing.
- The optical density of each well is measured within 5 minutes, using a micro-plate reader, set to 450 nm.
- The reader is set again to 540 nm and a second measurement is taken.
- The readings at 540 nm is subtracted from the readings at 470 nm. (this allows correction for optical imperfections in the plate).

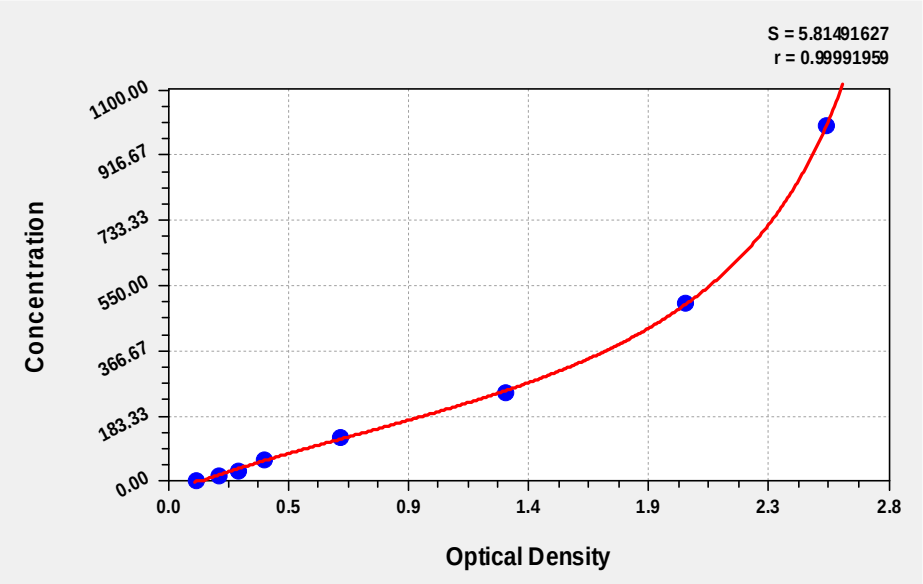
Calculation:

A standard curve was constructed by plotting the concentration on the x-axis and the mean absorbance of each standard on the Y-axis and a best fit curve was drawn through points on the graph.

The following table shows the absorbance (OD) of each standard and the concentration of CK 18M30 in the sample was obtained based on this absorbance.

Standard(mIU/mL）	O.D
1000	2.546
500	2.003
250	1.314
125	0.674
62.5	0.382
31.2	0.281
15.6	0.206
0	0.121

STANDARD CURVE OF CK 18M30



Reference range:

Healthy : < 150 U/L

Slightly elevated : 150 – 200 U/L (indication for mild fibrosis as in NAFL)

Elevated : > 200 U/L (substantial fibrosis as in NASH)

ANTHROPOMETRIC MEASUREMENTS

Height is measured in meters,weight is measured in kilograms and Body mass index is calculated as:

$$\frac{\text{Weight (kg)}}{\text{Height (m}^2\text{)}}$$

It has been used as an estimate of overall adiposity.

Normal weight - 18.5 to 24.5

Overweight - 25 to 29.9

Obesity > 30

ANALYTES

Automated chemistry analyzer ERBA 640 was first calibrated using the XL MULTICAL calibrator traceable to Isotope dilution mass spectrometry(IDMS). Plasma glucose,total cholesterol, triglycerides,HDL uric acid,AST and ALT were analysed using ERBA 640 automated analyser.. Results were calculated automatically by the instrument

ESTIMATION OF PLASMA GLUCOSE

Method: Glucose Oxidase - Peroxidase Method (GOD – POD) End point assay

Glucose in the sample is oxidized to gluconic acid and hydrogen peroxide in the presence of Glucose oxidase. The enzyme peroxidasecatalyses the oxidative coupling of 4-aminoantipyrine with phenol to yield a colouredquinonemine complex.The intensity of the colourisdirectly proportional to the concentration of glucose in sample.

$$\beta\text{-D-Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{phenol} + 4\text{AAP} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 2H_2O$$

4AAP – 4 amino antipyrine.

REAGENT COMPOSITION

ACTIVE INGREDIENT	CONCENTRATION
PHOSPHATE BUFFER	250 mmol/L
GLUCOSE OXIDASE	25 U/Ml
PEROXIDASE	>2 U/mL
PHENOL	5 mmol/L
4 - AMINOANTIPYRINE	0.5 mmol/L

Procedure:

Automated chemistry analyzer ERBA 640 was first calibrated using the XL MULTICAL calibrator traceable to Isotope dilution mass spectrometry(IDMS) . Results were calculated automatically by the instrument Reference interval: Fasting plasma Glucose = 70-100 mg/dL.

ESTIMATION OF TOTAL CHOLESTEROL :

Method : Cholesterol esterase – cholesterol oxidase

End point assay

PRINCIPLE

$$\text{Cholesterol ester} + H_2o \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Fatty acids.}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol Oxidase}} \text{Cholestenone} + H_2O_2$$

$$2H_2O_2 + 4AAP + \xrightarrow{\text{Peroxidase}} \text{Phenolquinoneimine dye} + 4\ H_2O.$$

REAGENT COMPOSITION

REAGENT 1

ACTIVE INGREDIENT	CONCENTRATION
GOOD' S BUFFER	50 mmol/L
PHENOL	5 mmol/L
4 AMINO ANTIPYRINE	0.3 mmol/L
CHOLESTEROL ESTERASE	≥ 200 U/L
CHOLESTEROL OXIDASE	>50 U/L
PEROXIDASE	>3 kU/L

Reference interval:

Desirable : <200mg/dL

Borderline high : 200-230 mg/dL

High : >239 mg/dL

TRIGLYCERIDE:

Method : Enzymatic colorimetric method

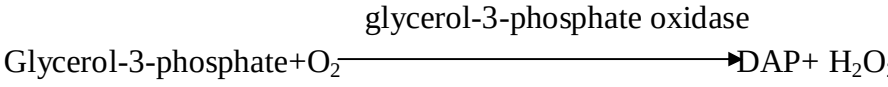
End point assay

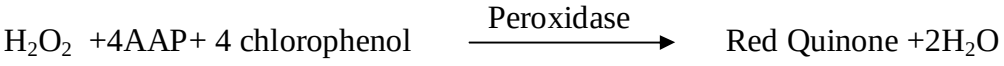
PRINCIPLE

The series of reactions involved in the assay system is as follows:

$$TGL + H_2O \xrightarrow{\text{Lipoprotein Lipase}} \text{glycerol+ free fatty acids.}$$

$$\text{Glycerol+ ATP} \xrightarrow{\text{Glycerol Kinase}} \text{glycerol-3-phosphate +ADP}$$





*DAP – Dihydroxy acetone phosphate

REAGENT COMPOSITION

Active Ingredient	Concentration
Good's buffer (pH 7.2)	50 mmol/L
4-Chlorophenol	4 mmol/L
Mg^{2+}	15 mmol/L
ATP	2 mmol/L
Glycerolkinase	≥ 0.4 KU/L
Peroxidase	≥ 2.0 KU/L
Lipoproteinlipase	≥ 2.0 KU/L
Glycerol-3-phosphate-Oxidase	≥ 0.5 KU/L
4-Aminoantipyrine	0.5 mmol/L

Reference values:

Males:60-165mg/dL

Females:40-140mg/dL.

Estimation of HDL-Cholesterol:

Principle:

The assay is based on a modified polyvinyl sulfonic acid (PVS) and polyethylene-glycol-methyl ether (PEGME) coupled classic precipitation method with the improvements in using optimized quantities of PVS/PEGME

and selected detergents.LDL, VLDL and chylomicron (CM) react with PVS and PEGME and the reaction results in inaccessibility of LDL, VLDL and CM by cholesterol oxidase (CHOD) and cholesterol esterase (CHER).

The enzymes selectively react with HDL to produce H_2O_2 which is detected through a Trinder reaction.

$$\text{HDL+LDL+VLDL+CM} \xrightarrow[\text{PEGME}]{\text{PVS}} \text{(LDL + VLDL + CM) + PVS/PEGME}$$

$$\text{HDL} \xrightarrow{\text{CHOD, CHER}} \text{Fatty Acid} + H_2O_2$$

$$2H_2O_2 + \text{4-AA+TODB} \xrightarrow{\text{Peroxidase}} \text{Quinone} + 5\ H_2O$$

REAGENT COMPOSITION

R1

Active Ingredient	Concentration
MES buffer (pH 6.5)	6.5 mmol/L
TODB N, N-Bis(4-sulfobutyl-3-methylaniline)	3 mmol/L
Polyvinyl sulfonic acid	50 mg/L
Polyethylene-glycol-methyl ester	30 mg/L
$MgCl_2$-	2 mmol/L

R2

Active Ingredient	Concentration
MES buffer (pH 6.5)	50 mmol/L
Cholesterol esterase	5 kU/L
Cholesterol oxidase	20 kU/L
4-aminoantipyrine	0.9 g/L
Detergent	0.5 %

Reference interval:

Low: <40 mg/dL

High > 50 mg/dL

URIC ACID ESTIMATION:

PRINCIPLE

End point assay

This reagent is based onTrinder reaction. The following are the reactions involved .

$$\text{Uric Acid} + O_2 + H_2O \xrightarrow{\text{Uricase}} \text{Allantoin} + CO_2 + H_2O_2$$

$$\text{TOOS} + 4\text{AAP} + 2H_2O_2 \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + 4H_2O$$

REAGENT COMPOSITION

R1

Active Ingredient	Concentration
Pipes Buffer (pH 7.0)	50 mmol/L
4-Aminoantipyrine	0.375 mmol/L
Uricase	≥200 U/L

R2

Active Ingredient	Concentration
Pipes Buffer (pH 7.0)	50 mmol/L
TOOS	1.92 mmol/L
Peroxidase	≥5000 U/L

Reference interval:

Male: 4.4 to 7.6 mg/dL

Female: 2.3 to 6.6 mg/dL

ALT

PRINCIPLE

This ALT/GPT reagent is based on the recommendations of the IFCC without pyridoxal phosphate.

ALT/GPT
L-Alanine + 2-oxoglutarate ⟶ Pyruvate + L-Glutamate

LDH
Pyruvate + NADH ⟶ L-Lactate + NAD

LDH
Sample pyruvate + NADH ⟶ L-Lactate + NAD

REAGENT COMPOSITION

R1

Active Ingredient	**Concentration**
Tris buffer (pH 7.5)	137.5 mmol/L
L-Alanine	709 mmol/L
LDH (microbial)	≥ 2000 U/L

R2

Active Ingredient	**Concentration**
CAPSO	20 mmol/L
2-oxoglutarate	85 mmol/L
NADH	1.05 mmol/L

Reference interval:

Male: <45U/L

Female: <34U/L

ESTIMATION OF AST:

PRINCIPLE

This reagent is based on IFCC recommendations, without pyridoxal phosphate.

The series of reactions involved in the assay system is as follows:

AST/GOT
L-Aspartate + 2-oxoglutarate ⟶ Oxaloacetate + L-Glutamate

MDH
Oxaloacetate + NADH ⟶ Malate + NAD

LDH

Sample pyruvate + NADH ⟶ L-lactate + NAD

REAGENT COMPOSITION

R1

Active Ingredient	**Concentration**
Tris buffer (pH 7.8)	110 mmol/L
L-aspartic acid	340 mmol/L
LDH	≥ 4000 U/L

R2

Active Ingredient	**Concentration**
CAPSO	20 mmol/L
2-oxoglutarate	85 mmol/L
NADH	1.05 mmol/L

Reference interval:

Male: <35 U/L

Female :<31U/L

ULTRASOUND ABDOMEN

Ultrasound abdomen has been performed for all patients to find out the echogenic pattern characteristic of visceral fatty liver.The patients are graded as having grade 1, grade II or grade III fatty liver with (or) without hepatomegaly.

TRANSIENT ELASTOGRAPHY (FIBROSCAN)

Fibroscan was performed for those patients diagnosed as fatty liver on ultrasound and those patients with elevated AST and ALT levels.This was performed with a special probe which was placed in the right upper quadrant of the abdomen.This measured the fibrosis of the liver (stiffness of the liver) in kilopascals.

A value of < 7 kpa is normal.

7-13kpa fibrosis.

> 13kpa cirrhosis.

Statistical analysis

STATISTICAL ANALYSIS

- Data was analyzed using SPSS software version 16.0 and P value less than 0.05 was considered as statistically significant.
- BMI, total cholesterol,triglycerides ,HDL, plasma glucose and uric acid were compared between study groups by Student's t-test.
- The association of BMI,Total cholesterol, Triglycerides ,HDL, and Uric acid, with cytokeratin 18M30 were studied by Pearsons correlation method.
- One way ANOVA was done to compare more than two variables in the same group & between two group . It was carried out to compare Cytokeratin 18 M30 between NAFL& NASH patients.
- Receiver operating characteristics curve analysis was done to assess the utility of cytokeratin 18M30 in the diagnosis of NASH.
- Serum Cytokeratin 18M30 levels between the groups were analyzed by regression analysis and scatter diagram plotted.

Results

Master Chart of NAFL Patients																
Sno	Age	Gender	Height cm	Weight kg	BMI kg/m^2	CK18M30 U/L	Fastng PG mg/dL	Total cholesterol mg/dL	TGL mg/dL	HDL mg/dL	Uric Acid mg/dL	AST U/L	ALT U/L	Utrasonogram	Fibro Scan	Diabetes mellitus
1	56	M	156	72	29.59	184.56	87	156	145.3	38	6.1	85	48	FL	6	Y
2	33	F	149	52	23.42	198.99	94	208	179	39.4	6	43	31	FL	6	N
3	56	F	143	61	29.83	187.67	114	206	122.2	33	4.8	48	38	FL	6	N
4	57	F	145	56	26.63	189.14	87	189	198.4	27.3	6.3	17	30	FL	6	N
5	30	F	154	50	21.08	219.56	108	212	117.3	43	3.91	81	20	FL	7	Y
6	44	M	162	61	23.24	159.34	99	186	145	47	5.7	49	53	FL	2	N
7	56	F	142	62	29.09	165.89	110	193	140.4	35.7	3.9	49	67	FL	3	Y
8	64	M	158	65	26.04	178.34	90	188	210	26.8	10.56	45	47	FL	6	N
9	61	F	162	70	26.67	185.14	111	201	221	31.6	3.45	33	44	FL	6	Y
10	42	F	140	54	27.55	202.45	101	229	194	27.8	8.15	29	28	FL	7	N
11	54	F	144	72	34.72	201.87	116	206	126	32	5.9	48	84	FL	7	Y
12	53	F	148	65	29.67	189.18	108	151	162.4	49.6	4.8	45	22	FL	6	N
13	48	F	154	56	27.83	187.34	98	156	145.5	34.5	4.6	19	29	FL	6	N
14	31	F	135	44	24.14	167.43	99	126	71.8	47.8	2.34	23	51	FL	3	N
15	35	F	143	53	25.92	197.56	87	146	133.4	42.4	4.2	19	89	FL	6	N
16	56	M	159	68	26.9	188.45	89	214	168	42	7.25	32	24	FL	6	Y
17	36	F	138	36	30.46	168.11	89	153	186	39	5.3	36	33	FL	4	N
18	58	M	161	70	27.01	198.56	103	156	118	52.5	6.5	56	29	FL	6	N
19	26	M	151	68	29.8	176.89	99	231	278.9	47.3	6.3	31	24	FL	5	N
20	47	M	151	62	27.19	185.56	113	167	143	38	7.2	34	27	FL	6	Y
21	48	M	159	68	26.9	162.11	67	117	111.4	51	6.9	28	35	FL	3	N
22	29	M	143	52	25.43	150.23	95	127	125	32	4.78	45	47	FL	2	N
23	60	F	153	68	29.05	168.95	89	162	167	43	5.1	56	11	FL	4	N
24	43	M	153	65	27.77	156.45	98	158	132.5	44.5	4.34	67	32	FL	2	N
25	68	F	153	60	25.63	178.87	87	242	125.9	27.8	1.84	45	30	FL	6	N
26	63	M	142	65	32.24	167.54	122	231	278.9	47.3	5.4	49	29	FL	3	N
27	45	F	154	62	26.14	155.87	97	143	110.5	41	2.3	30	46	FL	2	N
28	45	F	155	65	27.06	176.98	97	153	147	38.1	3.4	29	10	FL	5	N
29	69	F	151	72	23.24	153.14	109	152	183.7	41.8	5.3	29	19	FL	2	N
30	60	M	163	66	24.84	151.64	97	156	143.8	42	5.34	34	58	FL	2	N
31	61	M	165	70	25.71	175.61	117	168	155	41.5	5.3	37	18	FL	5	N
32	52	F	156	59	24.24	204.45	124	168	154	38	6	15	38	FL	7	N
33	40	M	159	61	24.13	197.67	118	210	165	29	7.1	34	46	FL	6	N
34	55	F	142	54	26.78	194.45	111	206	147.8	28	4.9	29	30	FL	6	N
35	54	M	160	74	28.01	168.45	107	235	198.5	41.3	4.6	22	51	FL	4	N

FL - Fatty liver

Master Chart of NASH Patients

Sno	Age	Gender	Height cm	Weight kg	BMI kg/m^2	CK18M30 U/L	Fastng PG mg/dL	Total cholesterol mg/dL	TGL mg/dL	HDL mg/dL	Uric Acid mg/dL	AST U/L	ALT U/L	AST/ALT	Utrasonogram	Fibro Scan	Diabetes mellitus
1	43	F	148	59	26.94	263.87	96	244	222.5	25.4	6.8	15	33	0.93	FL	8	N
2	64	M	146	60	28.15	498.45	131	231	220.5	26.5	6.4	48	94	0.93	FL	11	Y
3	68	F	145	67	31.87	705.89	142	248	242.5	23.2	3.4	29	30	1.17	FL	20	N
4	49	M	146	61	28.62	504.67	100	248	239.8	26.8	6.1	34	43	0.97	FL	11	Y
5	54	M	157	68	27.59	554.38	107	196	187.5	31.5	6.8	22	87	1.19	FL	13	N
6	57	M	161	75	28.93	664.98	80	356	388.6	19.6	5.6	39	35	1.13	FL	16	N
7	56	F	150	61	27.11	668.56	129	178	181.3	29.6	4.9	21	28	1.10	FL	16	Y
8	34	M	158	70	28.04	487.65	116	196	201.4	31.5	14.3	24	34	0.85	FL	11	Y
9	46	M	162	68	25.91	330.78	94	178	165.3	37.8	6.1	35	54	0.89	FL	9	Y
10	49	F	140	68	34.69	765.64	134	276	323	24.4	9.8	30	34	1.19	FL	24	Y
11	56	F	148	71	32.41	239.78	123	179	163.6	42.6	4.9	29	39	0.83	FL	8	Y
12	44	M	142	70	34.72	226.67	129	246	198.6	23.1	8.4	23	49	0.88	FL	8	Y
13	56	F	159	69	27.29	553.87	99	208	256	25.8	3.4	28	28	0.62	FL	12	N
14	43	M	164	79	29.37	605.89	96	303	288.5	22.7	14.3	30	30	1.33	FL	15	N
15	53	M	169	74	25.91	443.35	99	187	198.5	26.7	5.8	26	31	0.82	FL	10	N
16	58	M	150	64	28.44	456.85	110	258	232.5	23.2	7.3	42	49	0.92	FL	10	N
17	42	M	164	82	30.49	677.98	124	298	288.8	20.8	5.8	56	29	1.09	FL	17	Y
18	40	F	153	61	26.05	276.67	87	167	144.4	47.3	2.5	56	64	0.89	FL	8	N
19	56	F	149	64	28.83	705.45	91	167	156.5	45.8	5.2	48	17	1.12	FL	20	N
20	60	M	153	65	27.77	453.87	115	267	256	27.1	5.7	76	20	0.92	FL	10	Y
21	67	M	157	75	30.43	774.87	133	278	365.5	21.2	6.1	33	58	1.19	FL	25	Y
22	57	F	158	65	26.04	233.14	93	178	156	38.1	6.1	29	23	0.89	FL	8	Y
23	62	F	142	65	32.24	554.89	99	248	242.5	23.2	5.2	47	32	1.08	FL	13	N
24	55	F	151	68	29.82	450.67	120	229	198.5	21.4	4.9	34	50	0.93	FL	10	Y
25	50	M	159	68	26.9	331.78	115	211	208	27.8	5.8	76	78	0.46	FL	9	Y

FL - Fatty Liver

Master Chart of Controls Group														
Sno	Age	Gender	Height cm	Weight kg	BMI kg/m^2	CK18M30 U/L	Fastng PG mg/dL	Total cholesterol mg/dL	TGL mg/dL	HDL mg/dL	Uric Acid mg/dL	AST U/L	ALT U/L	Utrasono gram
1	23	F	143	43	21.03	64.45	89	164	173.3	38.8	4.9	23	11	N
2	34	M	149	50	22.52	78.56	91	156	167	43.7	3	33	38	N
3	36	M	158	59	23.63	84.45	108	183	164	51.8	4.1	29	25	N
4	40	M	154	56	23.61	78.76	104	190	75.7	65.9	5.1	39	29	N
5	28	F	146	51	23.93	78.87	99	170	75	50.6	4.9	21	31	N
6	44	M	154	55	23.19	83.54	102	164	167.3	52.8	2.67	30	26	N
7	20	M	150	56	24.89	83.56	94	176	178.5	34.4	6.3	29	29	N
8	35	F	151	51	22.37	77.86	101	178	165.4	51.8	1.9	19	10	N
9	33	F	155	54	22.48	67.57	76	176	156.4	39.8	5.2	31	29	N
10	38	M	161	64	24.69	88.67	101	163	134.8	45.8	2.3	34	19	N
11	38	M	149	48	21.62	63.86	75	184	86	42.3	3.8	32	28	N
12	52	F	148	44	20.09	56.56	71	141	114.1	34.5	4.9	31	13	N
13	29	M	159	60	23.73	78.87	78	174	162.8	42.5	4.76	33	20	N
14	56	M	154	58	24.46	82.58	98	146	154.3	42.7	4.5	10	43	N
15	34	F	156	55	22.6	75.67	89	168	144.4	48.7	1.78	28	32	N
16	34	F	149	52	23.42	77.65	94	143	177.7	36.7	2.96	26	19	N
17	30	F	151	55	24.12	83.98	107	193	140.4	52.4	4.5	19	44	N
18	37	M	152	50	21.64	64.56	68	174	163.5	40.1	4.7	35	38	N
19	33	M	158	58	23.23	84.01	95	145	110.8	56.5	3.4	34	20	N
20	37	F	153	54	23.07	76.56	65	167	175.5	38.6	5.4	22	34	N
21	31	F	147	58	26.84	94.76	60	189	151.5	42.6	3.56	13	23	N
22	38	M	150	46	20.44	56.87	98	148	148.3	52.5	2.46	39	29	N
23	56	M	155	55	22.89	80.03	84	139	145.6	38.9	4.1	29	36	N
24	33	F	142	48	23.8	76.45	97	178	165.6	54.3	1.78	20	19	N
25	37	M	148	50	22.83	68.86	89	154	163	38.7	4.9	28	38	N

N - Normal

RESULTS OF THE STUDY

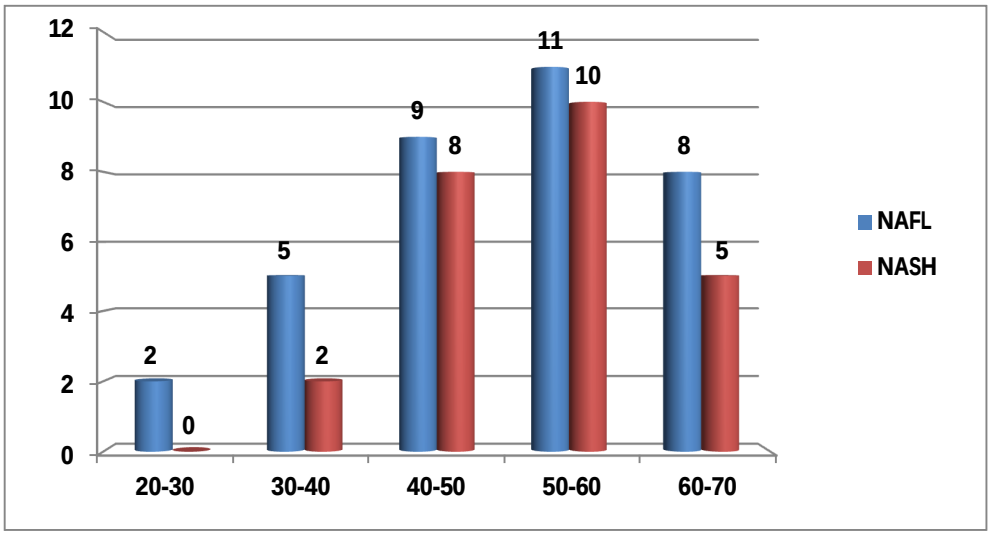
Table:1 Comparison of age between NAFL and NASH

Age	NAFL	NASH
20 – 30	2	0
30-40	5	2
40 –50	9	8
50 – 60	11	10
60 – 70	8	5

There were 9 ,11 and 8 patients of NAFL in the fourth ,fifth and sixth decades.

There were 2,8 and 10 patients of NASH in the fourth,fifth and sixth decades.

Figure 10:Bar diagram showing comparison of age between NAFL and NASH



Occurrence of NASH and NAFL is much higher in 40-50, 50-60 and 60-70 age group compared to 20 -30 and 30-40 age group.

Table 2 :BMI in NAFL and NASH compared to healthy controls.

	No	Mean	Standard Deviation	Standard Error of Mean	p value
NASH	25	28.98	2.56	.512	0.004
NAFL	35	25.81	1.67	.282	
CONTROLS	25	24.22	1.08	.217	

p value 0.004

TABLE 2: shows the comparison of BMI between NAFL ,NASH and healthy controls. The mean BMI in the control group was 24.22 (+/-1.08) and in the NAFL group was 25.81 (+/- 1.67) and in the NASH group was 28.98 (+/- 2.56). A significant p valueof 0.004was obtained.

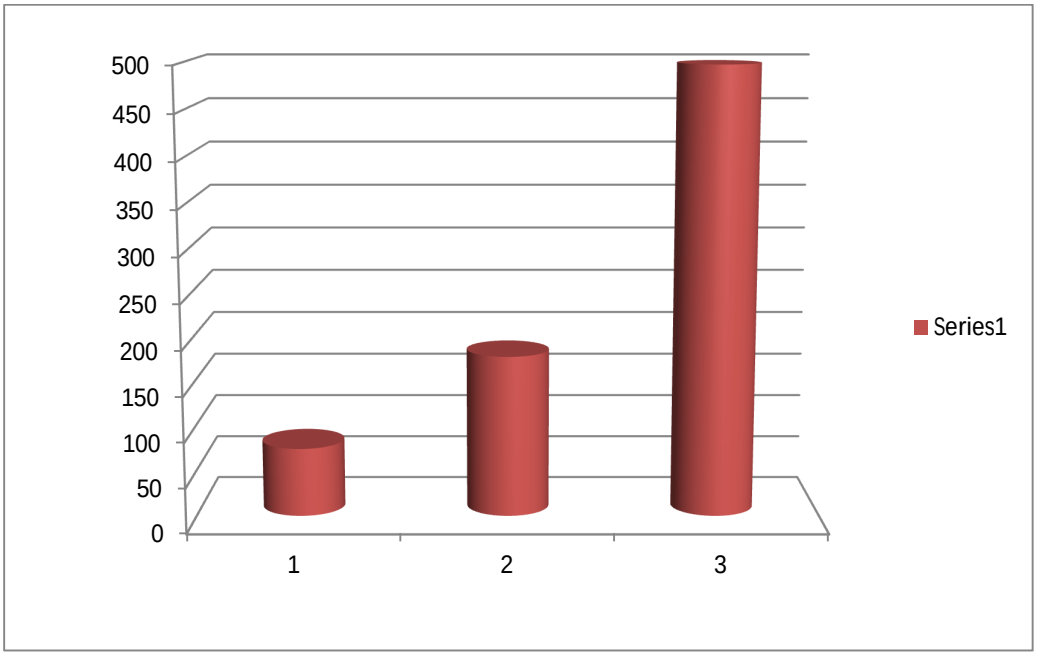
Table 3 :Comparison of cytokeratin 18m30 in NAFL and NASH with healthy controls - anova

	No.	MEAN	STANDARD DEVIATION	STANDARD ERROR OF MEAN	p value
CONTROLS	25	76.30	9.56	1.91	< 0.01
NAFL	35	179.84	17.45	2.94	
NASH	25	497.22	173.67	34.73	

Usinganovatest,TABLE – 3 shows Cytokeratin 18M30 in various stages of NAFLD including NAFL and NASH compared to healthy controls.

The mean Cytokeratin 18 M30 in NAFL was found to be 179.84 (+/- 17.45) and in NASH was 497.22 (+/- 173.67) and in the control group was 76.30 (+/- 9.56).There was statistically significant difference in CK 18M30 levels between the three groups with the highest being in NASH compared to NAFL and controls.A highly significant p value of <0.01 was obtained .

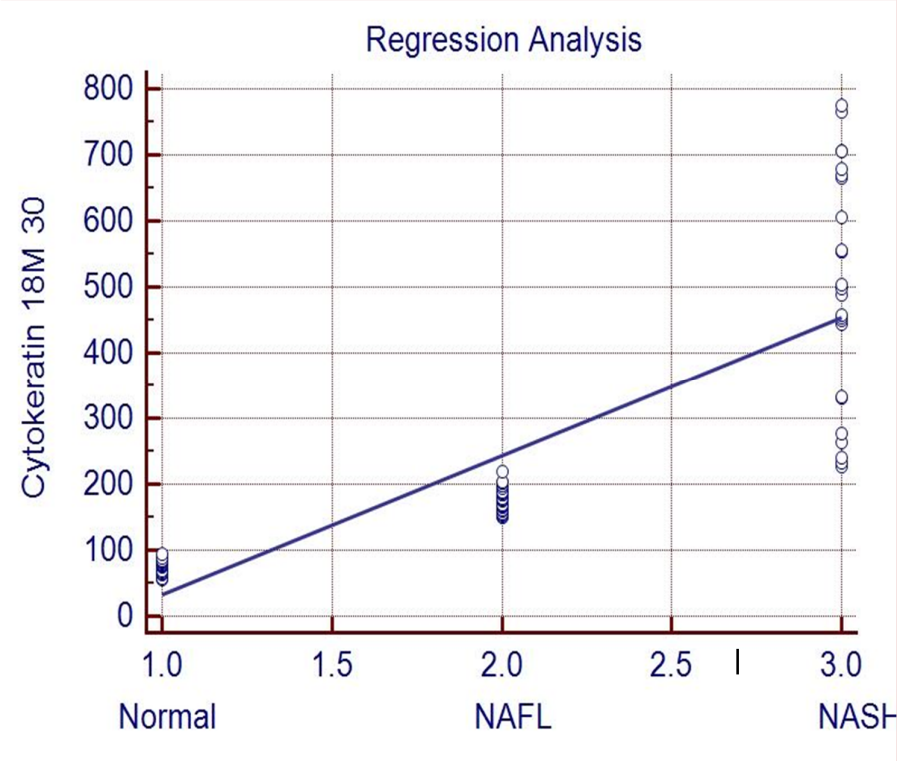
Figure11: Bar diagram of cytokeratin 18m30 levels in NASH, NAFL compared to controls



1.Controls 2.NAFL 3. NASH

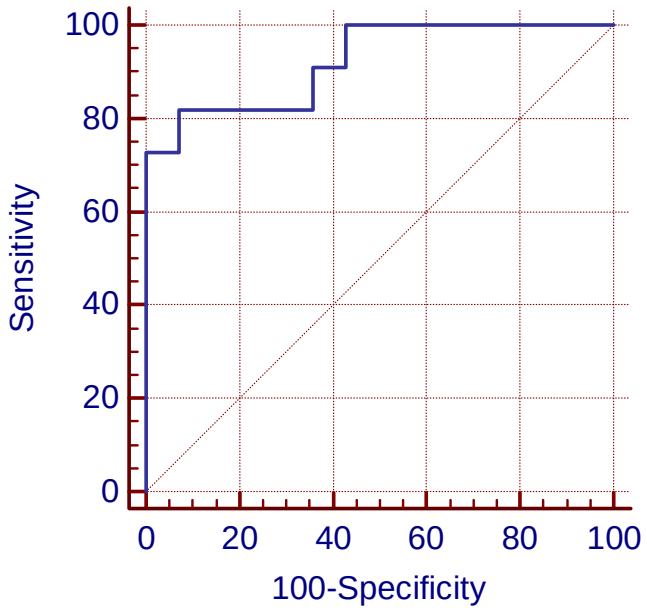
Cytokeratin 18M30 levels are greatly elevated in NASH compared to NAFL and controls.

Figure 12:Regression analysis - scatter plot of cytokeratin 18m3o in NASH, NAFL and controls



Scatter plot shows linear plot with positive gradient of cytokeratin 18M30.It also shows highly elevated levels of cytokeratin 18M30 in NASH compared to NAFL and controls.

Figure13: ROC (receiver operating characteristics) curve for cytokeratin 18m30 in NASH



ROC curve plotted between cytokeratin 18M30 levels and fibroscan in NASH shows specificity of 92.9 % (highly specific), sensitivity of 81.8%(moderately specific), at a cut off point of 553.87U/L. Area under the curve (AUROC) is 0.922, with a highly significant p value of < 0.01(95% CI of 0.742 to 0.990).

TABLE 4 :Lipid profile in NAFL, NASH and healthy controls

		N	MEAN	STANDARD DEVIATION	STANDARD ERROR OF MEAN	p value
TOTAL CHOLESTEROL	CONTROLS	25	158.92	15.99	3.19	0.019
	NAFL	35	217.54	46.34	7.83	
	NASH	25	225.88	27.40	5.48	
TRIGLYCERIDES	CONTROLS	25	146.43	30.78	6.15	0.007
	NAFL	35	175.98	34.42	5.81	
	NASH	25	178.23	18.57	3.71	
HDL	CONTROLS	25	45.49	7.86	1.58	0.002
	NAFL	35	38.67	5.81	0.98	
	NASH	25	28.40	7.64	1.52	

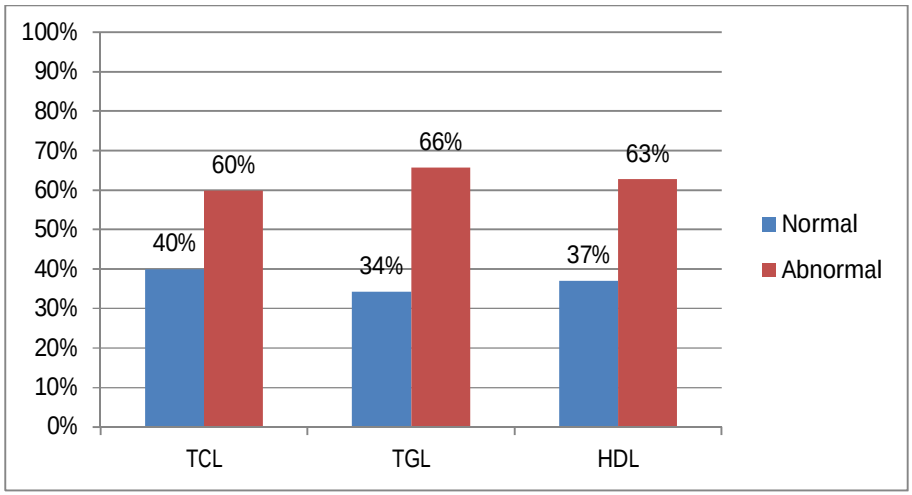
TABLE 4: shows the data Total cholesterol,Triglycerides and HDL in NAFL , NASH compared to healthy controls.

A significant p value was obtained for Total cholesterol, Triglycerides and HDL.

1. The meantotal cholesterol in NAFL was (217.54+/-46.34) and inNASHwas (225.88 +/- 27.40) with a p value of 0.019.
2. The mean triglycerides in NAFL was (175.98 +/- 34.42) and in NASH was (178.23+/- 18.57) with a p value of 0.007.
3. The mean HDL in NAFL was (38.67 +/- 5.81) and in NASH (28.40 +/- 7.64) with a p value of 0.002.

This table shows statistically significant difference between NASH and NAFL group, and NASH and control group regarding total cholesterol,triglycerides HDL.

Figure 14 :Shows normal and abnormal frequency of lipid profile in NAFL



Total cholesterol was elevated in 60% ,triglycerideswas elevated in 66% and HDL was decreased in 63% of NAFL cases.

Figure 15 : Shows normal and abnormal frequency of lipid profile in NASH

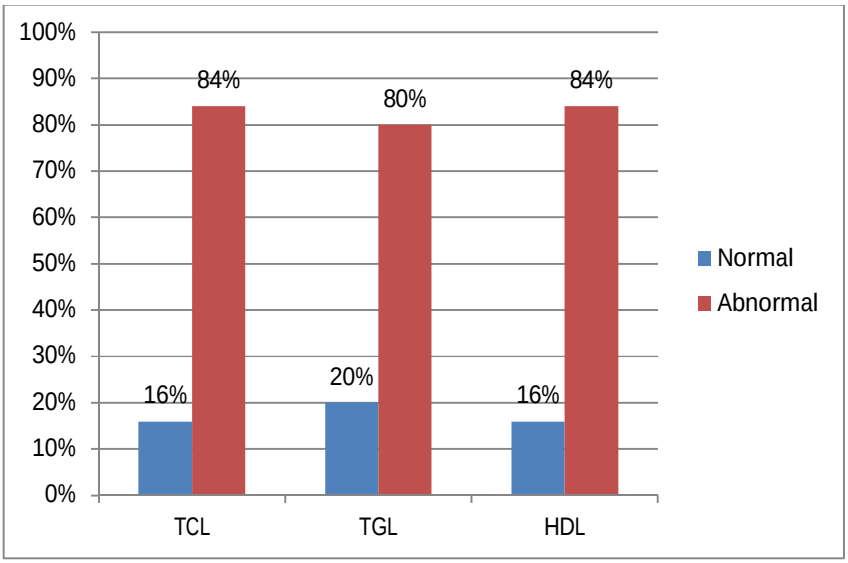


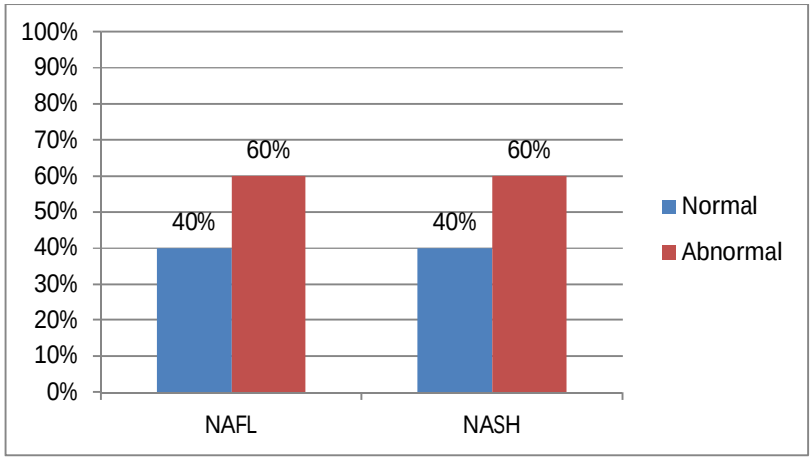
Figure 15 shows total cholesterol was increased in 84%, triglycerides was increased in 80% and HDL was decreased in 84 % of NASH cases.

Table 5 : Comparison of fasting plasma glucose levels between NAFL, NASH and controls

	No	Mean	Standard Deviation	Standard error of Mean	p value
CONTROLS	25	84.20	11.46	2.29	0.003
NAFL	35	104.86	10.57	1.78	
NASH	25	111.48	15.95	3.19	

The mean fasting blood plasma glucose was 84.20 (+/- 11.46) in the control group,104.86 (+/- 10.57) in the NAFL group and 111.48 (+/- 17.122) in the NASH group with a significant p value of 0.003.There was stastistically significant difference in FBS levels between NASH and NAFL group, and between NASH and control groups.

Figure 15: Shows frequency of normal and abnormal fasting plasma glucose in NASH and NAFL groups



Poor glycemic control was seen in 60% NAFL and 60% NASH cases.

Table 6: Comparison of uric acid between NAFL,NASH and controls

	No	Mean	Standard Deviation	Standard error of Mean	p value
CONTROLS	25	3.91	1.26	0.25	< 0.001
NAFL	35	6.33	1.19	0.20	
NASH	25	6.81	2.51	0.50	

The mean uric acid in the NASH group was 6.81(+/-1.19) and in the NAFL group was 6.33(+/- 2.51) and in the control group was 3.91(+/-1.26) with a highly significant p value of <0.001.There was statistically significant difference in uric acid levels between NASH and NAFL groups, and NASH and control groups.

Table 7 : Comparison of liver enzymes AST and ALT between NAFL NASH and controls

		No	Mean	Standard Deviation	Standard Error of Mean	p value
AST	CONTROLS	25	25.56	8.46	1.69	0.004
	NAFL	35	34.60	11.36	1.92	
	NASH	25	102.96	31.	6.20	
ALT	CONTROLS	25	32.52	8.58	1.71	0.001
	NAFL	35	46.86	14.23	2.40	
	NASH	25	109.60	27.19	5.43	

TABLE 7: AST, ALT and ALP were compared between the study groups.The mean AST in NAFL was (34.60+/-11.36) and in NASH (102.96+/-31)with a significant p value of 0.004 . The mean ALT in NAFL was (46.86+/-

14.23) and in NASH (109.60+/- 27.19) with a significant p value of 0.001.There was stastistically significant difference between NASH and control group and between NAFL and control groups in uric acid levels.

TABLE 8: Correlation between CK 18M30 and other parameters in NASH (Pearson's correlation).

	Cytokeratin 18M30	
	r value	**p value**
BMI	0.274	0.004
Total cholesterol	0.484	0.019
Triglycerides	0.251	0.007
HDL	0.201	0.002
Fasting plasma glucose	0.334	0.003
Uric acid	0.364	<0.001

Table 8: Pearson' s correlation shows statistically significant positive correlation between cytokeratin 18M30 and BMI , total cholesterol, triglycerides,HDL, fasting plasma glucose and uric acid in NASH.

Discussion

DISCUSSION

Non alcoholic fatty liver disease is the most common chronic liver disease especially in the developed and industrialized countries. NASH forms part of thedisease which encompasses non alcoholic fatty liver (NAFL), NASH and NAFLD – related cirrhosis.About one fifth of patients with NASH progress to develop cirrhosis[115]. Once cirrhosis has occurred, 45% patients will develop serious complications such as variceal bleeding, ascites, and end stage liver failure. The most severe end of the spectrum is hepatocellular carcinoma.

The estimated prevalence of NAFLD is 9 - 37% of the general population and NASH occurs in 2% of the general population.

NAFLD is now recognized as an important component of metabolic syndrome, that makes an individual more prone for atherosclerotic cardiovascular disease complications. Most of the deaths in patients with the disease are mainly attributed to cardiovascular disease.

Yesui et al conducted a cumulative 5 year study and concluded that, NASH accounts for at least 11.3% of total cases of Hepatocellular carcinoma (HCC)[154]. There is recent evidence that there is high incidence of HCC in type 2 diabetics and this can beaccounted for thehigh prevalence of NASH in these cases.

All patients of simple fatty liver do not progress to NASH or cirrhosis, and patients with NASH may revert to simple fatty liver or even normal and

the severity of the degree of steatosis may come down if the condition is diagnosed early and therapeutic interventions are started early.

The gold standard to diagnose NAFLD is liver biopsy.But this is an invasive procedure which is cumbersome, and produces many complications in patients.The ultrasonography is able to pick up steatosis only when there is more than 20% fat deposition in the liver .Transient elastography (fibroscan) can diagnose NASH and fibrosis, but is not easily available.

So, when the disease is diagnosed at a very initial stage using a simple and easily available biomarker and lipid lowering agents instituted early, the progress of the disease can be curtailed . If modification of lifestyle is also initiated early by starting on exercise and consuming suitable low calorie diet, the severity of the disease can be reduced and the most dreaded complications can be prevented.

This study aimed at evaluating serum levels of Cytokeratin 18 M30 as a noninvasive biomarker in various stages of NAFLD.

In our study we included 3 groups –

- Healthy Controls - 25
- NAFL (non alcoholic fatty liver) - 35
- NASH (non alcoholicsteatohepatitis) - 25

In this study we evaluated serum Cytokeratin 18 M30 levels using sandwich ELISA technique .

Our study, included 25 NASH patients out of which 14 patients were males (56%)and 11(44%) were females, and 35 NAFL patients. Out of the 35 NAFL patients , 18 were males (51%) and 17 (49%) were females.

The mean age in NASH group was 49.36 (+/-10.34) and in NAFL group was 58.40 (+/-8.46) .This is similar to the conclusions by Koehler EM[155] thatNAFLD mainly affects the middle aged and the elderly because, the risk factors for its development,tend to increase with advancing age .In Indian studies mean age was reported to be 42.90 (+/- 10.54) by RoliAgarwal[156].

The mean BMI was greatly increased in NASH and NAFL groups when compared to the control group.Ruhl et al concluded that prevalence of NAFLD increases with increase in BMI[157].Prevalence studies have proved that the occurrence of the disease is about 60 – 70 % obese patients and it is closely linked with central adiposity.

In our study the CK 18 M 30 levels were found to be significantly higher in the NASH group with a mean of 497.22 (+/- 173.67) IU/ L and in the NAFL group it was 179.84 (+/-17.45) IU/L compared to the control group where the mean was 76.30 (+/- 9.569) IU/l. A highly significant p value of <0.01was obtained. There is statistically significant difference in CK 18M30 levels between the three groups with the highest being in NASH compared to NAFL and controls. . Hence,CK 18M30 levels can discriminate NASH from nonalcoholic fatty liver, as well as normal individuals.

This is similar to the study by Feldstein et al who proved that, CK18 M30 levels were increased in biopsy proven NASH patients[149] compared to NAFL and normal controls.

Wieckowska et al said that, CK 18 M30 levels could serve as a marker of hepatic inflammation. This is explained by the hepatocyte apoptosis which plays a key role in the pathogenesis of NAFLD and progression of NAFLD to NASH[158].Hepatocyte apoptosis is mediated by caspase 3 which cleaves the cytokeratin 18 of NAFLD patients and is released into the sera. The magnitude of apoptosis correlates with the degree of liver damage and fibrosis. In this study,CK 18 M30 levels also increased with increasing grades of fibrosis detected by fibroscan.So, CK 18 M30 can noninvasively grade the severity of NASH. Feldstein et al concluded that, For every 10 U/L increase in CK 18M30 levels the possibility of diagnosing NASH increased by 70%[159].

In this study,the regression analysis and scatter plot shows positive prediction of CK 18M30 in the diagnosis of NASH and NAFL.

In this study, the ROC curve shows diagnostic performance of CK 18M30 with a high specificity of 92.9 % and moderate sensitivity of81.8% at a cut off point of553.87U/L with AUROC of 0.922 (p value of<0.01) in NASH.**It may be considered that, CK 18M30 is a highly specific and moderately sensitive marker in the diagnosis of NASH**.

Younossi et al proved that, " CK 18M30 is an independent predictor of NASH and CK 18M30 has sufficient to excellent, diagnostic accuracy, for the detection and exclusion of NASH in the setting of NAFLD[160]".

Assessment of the serum levels of CK 18 M30 can be a useful screening, diagnostic and staging biomarker for NASH, based on this study.

LIPID PROFILE

In this study, the total cholesterol was increased in 60% of NAFL cases and 84% of NASH cases ,the triglycerides was increased in 66% of NAFLand 80% of NASH cases,and HDL was decreased in 63% of NAFL and 84% of NASH cases.The total cholesterol,triglycerides and HDL were increased compared to the control group with highly significant p value for total cholesterol(0.019),triglycerides(0.007) and HDL (0.002).

In this study,there was positive stastistically significant correlation between CK 18M30 levels and total cholesterol (r value=.484) ,triglycerides (r value = .251) and HDL (r value = .201) .This was similar to the study by Miyassato et al who concluded that, there was significant correlation between CK 18 M30 and total cholesterol,triglycerides and HDL in NAFLD patients.

Bajaj et al proved that,subjects with NAFLD had higher levels of total cholesterol and serum triglycerides. Liver injury is caused by free cholesterol accumulation[161] .So serum lipid levels are able to predict the progression of NAFL and NASH.

In NAFLD patients, liver produces many atherogenic factors like cytokines and bad lipoproteins .So fatty liver is associated with increased triglycerides and total cholesterol combined with decreased HDL, which pose a threat for the development of cardiovascular disease. Hamaguchi et al implied that, NAFLD plays a central role in the increased incidence of CVD[(162)]

Cholesterol-rich atherogenic diet induces oxidative stress and provokes oxidative inflammation.Mari et al gave evidence that mitochondrial loading of free cholesterol, but not free fatty acids sensitize the liver to TNF – α induced steatohepatitis[(163)].

Lipid lowering agents have the potential to normalize these lipid levels.Lifestyle modification by starting on exercise, to produce weight loss and control obesity is another confirmed treatment for NAFLD.Restriction on the intake of calories reverses hepatic insulin resistance and steatosis in animal models .Fasting inhibits cholesterol and fatty acid synthesis and has protective effect on lipid metabolism.Two drugs which can be used for obesity treatment are orlistat,which reduces the intestinal fat absorption and sibutramine , an appetite suppressant.

Inhibition of cholesterol synthesis by statins alone, or by combination with antioxidants like vitamin E was found to be beneficial in treating dyslipidemicprofile.Vitamin E supplementation suppresses the lipid peroxidation and TNF- α gene expression.

Recent studies suggest that ezetimibe and fenofibrates inhibit intestinal cholesterol absorption.Dual inhibition of cholesterol absorption and synthesis by combined administration of ezetimibe and simvastatin is a highly efficacious lipid lowering strategy, and were shown to be effective and safe in NAFLD.

FASTING PLASMA GLUCOSE LEVELS

In this study, the fasting plasma glucose levels was increased in 60% of NAFL patients as well asNASH.The known cases of type 2 diabetes was 56% of NAFL patients and 67% of NASH patients.

In this study, there was a statistically positive significant correlation between CK 18M30 levels and fasting plasma glucose levels (r = 0.334).

Gupte.P. et al studied that NAFLD is commonly associated with type 2 diabetes mellitus[(164)].Since insulin resistance is the main underlying mechanism in NAFLD,insulinsensitisers may be used in treatment.Thiazolidinediones (proglitazone and rosiglitazone) and biguanides (metformin) are insulin sensitisers.Thiazolidinediones are PPAR – γagonists.Following treatment with proglitazone and rosiglitazone, serum AST,ALT levels are significantly decreased in NAFLD.

URIC ACID

In this study, the mean uric acid was 6.87 (+/- 2.51) in NASH and 6.33 (+/- 1.19) in NAFL, when compared to the control group 3.91 (+/- 1.26) with a highly significant p value of < 0.001 .This shows that uric acid levels were in the higher normal range in NASH and NAFL groups .

This was similar to the findings of Yourning et al in his study, in which he proved that increased serum uric acid levels in the higher normal range was seen in NAFLD and elevated serum uric acid is an independent risk factor for NAFLD.

Recent studies have proved that, uric acid is released from injured cells following inflammation, and in NAFLD, one of the important components of liver damage is cell death which causes release and accumulation of uric acid in the serum.Hyperuricemia is commonly seen in metabolic syndrome and is positively correlated with insulin resistance.

Conclusion

CONCLUSION

The present study was done with an aim to evaluate Cytokeratin 18M30 as a noninvasive biomarker for non alcoholic fatty liver disease and its correlation with lipid profile to assess cardiovascular risk.

From this study we conclude that,

1. Cytokeratin 18M30 is a promising screening, diagnostic and staging biomarker for NASH.
2. Cytokeratin 18M30 can be used as a simple, and non invasive biomarker instead of liver biopsy.
3. Cytokeratin 18 M30 positively correlates with increasing grades of fibrosis in NASH.
4. There is significant positive correlation of total cholesterol, triglycerides and high density lipoprotein with cytokeratin 18M 30.
5. There is significant positive correlation of fasting plasma glucose with cytokeratin 18 M30.
6. There is significant positive correlation between uric acid and cytokeratin 18M30.
7. Hypercholesterolemia and hypertriglyceridemia is associated with development of NAFLD.
8. Poor glycemic control is associated with increased risk for NAFLD.
9. Increased uric acid is an independent risk factor for NAFLD.

Limitation of the study

LIMITATIONS OF THE STUDY

1. This study was done without comparing with liver biopsy which is the gold standard for diagnosing NAFLD.
2. The sample size for NASH is very small.
3. There are no reference values for cytokeratin 18M30 for NAFL and NASH in our population.
4. Follow up after instituting therapeutic management could not be done.

Scope for further studies

SCOPE FOR FURTHER STUDIES

1. Cytokeratin 18M30 can be used as a simple, non invasive biomarker in the management of NAFLD, after establishing the reference range.
2. Early diagnosis of NAFLD and therapeutic intervention, can reduce cardiovascular risk and thus reduce morbidity.
3. Uric acid can be used as an independent prognostic factor in NAFLD.
4. Future studies may be undertaken to establish a correlation with insulin resistance, hyperlipidemia, oxidative stress and adaptive response in areas of research in obesity.
5. Studies may be conducted to establish the role of hepatic transcription factors and target genes in regulating lipid metabolism.

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Annexures

MADRAS MEDICAL COLLEGE, CHENNAI 600 003

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Telephone No.044 25305301
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CERTIFICATE OF APPROVAL

To
Dr.J.Vinodha
II Year PG in M.D.(Bio-Chemistry)
Madras Medical College/RGGGH
Chennai 600 003

Dear Dr.J.Vinodha,

The Institutional Ethics Committee has considered your request and approved your study titled **" EVALUATION OF SERUM CYTOKERATIN 18 M30 LEVELS IN CASES OF NON ALCOHOLIC FATTY LIVER DISEASE " - NO.12012016.**

The following members of Ethics Committee were present in the meeting hold on **12.01.2016** conducted at Madras Medical College, Chennai 3

1.Dr.C.Rajendran, MD.,	:Chairperson
2.Dr.R.Vimala,MD.,Dean,MMC,Ch-3	:Deputy Chairperson
3.Prof.Sudha Seshayyan,MD., Vice Principal,MMC,Ch-3	: Member Secretary
4.Prof.B.Vasanthi,MD.,Inst.of Pharmacology,MMC,Ch-3	: Member
5.Prof.P.Raghumani,MS, Dept.of Surgery,RGGGH,Ch-3	: Member
6.Prof.M.Saraswathi,MD.,Director, Inst.of Path,MMC,Ch-3	: Member
7.Tmt.J.Rajalakshmi, JAO,MMC, Ch-3	: Lay Person
8.Thiru S.Govindasamy, BA.,BL,High Court,Chennai	: Lawyer
9.Tmt.Arnold Saulina, MA.,MSW.,	:Social Scientist

We approve the proposal to be conducted in its presented form.

The Institutional Ethics Committee expects to be informed about the progress of the study and SAE occurring in the course of the study, any changes in the protocol and patients information/informed consent and asks to be provided a copy of the final report.

Member Secretary - Ethics Committee

MEMBER SECRETARY
INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE
CHENNAI-600 003

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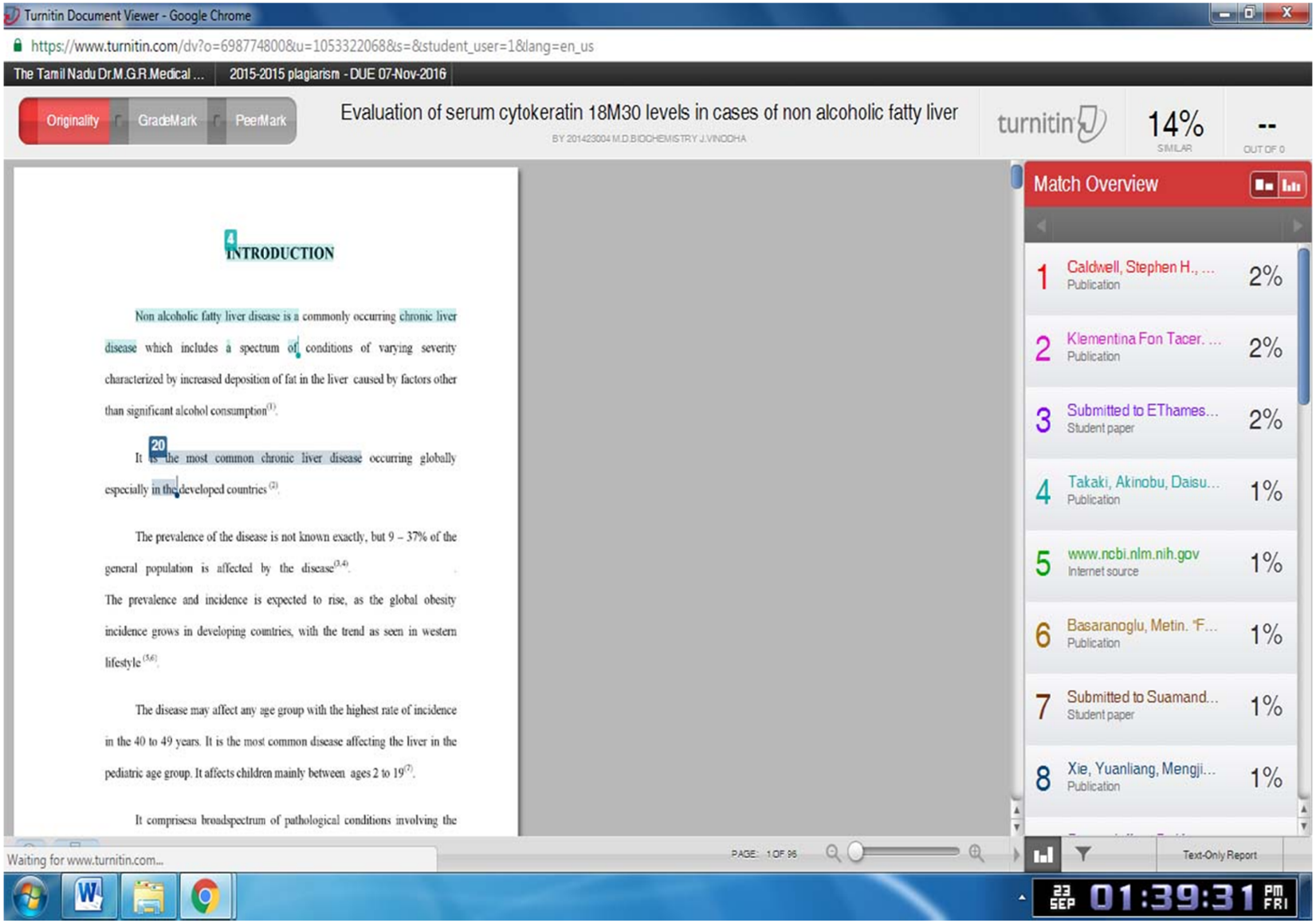
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INTRODUCTION
Non alcoholic fatty liver disease is a commonly occurring chronic liver disease which includes a spectrum of conditions of varying severity characterized by increased deposition of fat in the liver caused by factors other than significant alcohol consumption(1).
It is the most common chronic liver disease occurring globally especially in the developed countries (2).
The prevalence of the disease is not known exactly, but 9 – 37% of the general population is affected by the disease(3,4).
The prevalence and incidence is expected to rise, as the global obesity incidence grows in developing countries, with the trend as seen in western lifestyle (5,6).
The disease may affect any age group with the highest rate of incidence in the 40 to 49 years. It is the most common disease affecting the liver in the pediatric age group. It affects children mainly between ages 2 to 19(7).
It comprisesa broadspectrum of pathological conditions involving the
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INFORMATION SHEET

Title: Evaluation of serum Cytokeratin 18 M30 levels in cases of non alcoholic fatty liver disease.

Investigator : Dr. J. VINODHA,
II year Postgraduate,
Institute of Biochemistry,
Madras Medical College,
Chennai-600003.

Guide : Prof. Dr. R.CHITHRAA,
Institute of Biochemistry,
Madras Medical College,
Chennai-600003.

The purpose of the study is to evaluate the use of Cytokeratin 18 M30 as a non invasive biomarker in the diagnosis non alcoholic fatty liver disease.

Hence, I am doing this study titled “Evaluation of serum Cytokeratin 18 M30 levels in cases of non alcoholic fatty liver disease”, at Rajiv Gandhi Govt. General Hospital, Chennai. For this study I need 5mL of blood from 60 non alcoholic fatty liver disease patients and 30 apparently healthy individuals.

Your identity will be kept confidential throughout the study as well as during publication or presentation of the study findings in any clinical forum or journals. Participation in this study is purely voluntary. You can withdraw from this study at any time. Your decision will not result in any loss of benefits to which you are entitled. The results of the study will be intimated to you. If you have willingness to participate in this study, kindly sign in this information sheet and the consent form.

Signature of the investigator

Signature of the participant

Thumb impression

Place:

Date:

PATIENT CONSENT FORM

Title of the study **:"Evaluation of serum Cytokeratin 18 M30 levels in cases of non alcoholic fatty liver disease."**

Name : Date :
Age : OP No:
Sex : Project Patient No :

Documentation of the informed consent

I _____ have read the information in this form (or it has been read to me). I felt free to ask questions about the study which were answered. I, hereby, give my consent to be included as a participant in "**Evaluation of serum Cytokeratin 18 M30 levels in cases of non alcoholic fatty liver disease."**

1. I have read and understood this consent form and the information that was provided.
2. I have had the consent document explained.
3. I have been explained the nature of the study.
4. I have been explained about my rights and responsibilities by the investigator.
5. I have informed the investigator of all the treatments I am taking or have taken for the past -- months/years including any native (alternative) treatment.
6. I have been informed about the risks associated with my participation in this study.
7. I agree to cooperate with the investigator and I will inform him/her immediately if I suffer unusual symptoms.
8. I have not participated in any research study within the past _____ month(s).
9. I am aware of the fact that I can opt out of the study at any time without having to give my reason and this will not affect my future treatment in this hospital.
10. I am aware that the investigator may terminate my participation in the study at any time, for any reason, without my consent.
11. I hereby give permission to the investigator to release the information obtained from me as a result of participation in this study to the sponsors, regulatory authorities, Govt. agencies, and IEC. I understand that they are out of the interest of the public.

12. I have understood that my identity will be kept confidential even if my data are published.
13. I have had my questions answered to my satisfaction.
14. I have decided to be a participant in the research study.

I am aware that if I have any question during this study, I should contact the investigator. By signing this consent form, I attest that the information given in this document has been clearly explained. I have understood the contents of the consent form and, I will be given a copy of this document.

For participants:

Name and signature / thumb impression of the participant (or legal representative, if participant incompetent/For age 10-17 yrs-Name& signature of the parent/guardian.)

Name _____ Signature with date_____

Name and Signature of impartial witness (required for illiterate patients):

Name _____ Signature with date_____

Address and contact number of the impartial witness:

Name and Signature of the investigator or his representative obtaining consent:

Name _____ Signature with Date_____

ஆராய்ச்சி தகவல் தாள்

தலைப்பு:

"மதுபானம் சாரா கொழுப்பு கல்லீரல் நோயாளிகளைக் கண்டுபிடிப்பதில் சீரம்சைட்டோகெராட்டின் 18எம்30-ன் பங்கு பற்றிய ஆய்வு".

ஆராய்ச்சியாளர் : **மரு. J. விநோதா,**
பட்ட மேற்படிப்பு மருத்துவ மாணவி,
உயிர்வேதியியல் உயர்நிலைத்துறை,
சென்னை மருத்துவக் கல்லூரி மருத்துவமனை,
சென்னை - 600003.

ஆராய்ச்சி மேற்பார்வையாளர் : **பேரா. மரு. R.சித்ரா,**
உயிர்வேதியியல் உயர்நிலைத்துறை,
சென்னை மருத்துவக் கல்லூரி மருத்துவமனை,
சென்னை - 600003.

மதுபானம் சாரா கொழுப்பு கல்லீரல் நோய் நாள்பட்ட கல்லீரல் நோயாளிகளின் மத்தியில் பரவலாக ஏற்படும் நோய். அதற்கான காரணங்கள்:

1. நீரிழிவு நோய் (வகை 2)
2. மெட்டபாலிக் சின்ட்ரோம் (உடல் பருமனாக இருப்பது, இரத்தத்தில் கொழுப்பு சத்து அதிகமாக இருப்பது)
3. சில மருந்துகள் உட்கொள்வதனால் (ஸ்டீராய்டுகள், டமாக்சிஃபன், அமியோடரோன் போன்றவை)
4. மிகுதியான எடை குறைவதால் (இரைப்பை அறுவை சிகிச்சைகளால், மிகுதியான பட்டினியால்)
5. தைராய்டு சுரப்பி குறைவதனால்,

மதுபானம் சாரா கொழுப்பு கல்லீரல் நோயாளிகள் ஆண்கள் - 30 கிராம் (ஒவ்வொரு நாளும்), பெண்கள்-20 கிராம் (ஒவ்வொரு நாளும்) கடந்த 5 வருடங்களுக்கு மதுபானம் உட்கொள்ளாதவர்களாக இருக்க வேண்டும்.

மதுபானம் சாரா கொழுப்பு கல்லீரல் நோய் வெறும் கொழுப்பு சேர்ந்த கல்லீரல் அணுக்களாக இருக்கலாம் அல்லது கல்லீரல் இழைநார் வளர்ச்சி உள்ள கல்லீரல் நோயாக இருக்கலாம்.

கல்லீரல் நோய்களைக் கண்டுபிடிப்பதற்கு திசு ஆய்வு தான் தங்கத் தரநிலையாக நிலவி வருகிறது. ஆனால் திசு ஆய்வு ஒரு துளைத்தல் செயல்முறையாகவும், பல பக்க

விளைவுகள் உண்டாக்குவதாகவும் இருப்பதால் ஒரு துளைத்தல் இல்லாத செயல்முறை மிகுந்த மருத்துவ முக்கியத்துவமாக விளங்கும்.

சீரம்சைட்டோகெராட்டின் 18எம்30 ஒரு துளைத்தல் இல்லாத செயல்முறையாக மதுபானம் சாரா கொழுப்பு கல்லீரல் நோயாளிகளுக்கு மிகுந்த பயனுள்ளதாக இருக்கும்.

சைட்டோகெராட்டின் 18 கல்லீரல் செல்களில் உள்ள இடைநிலை இழை. கல்லீரல் செல்கள் விஷத்தன்மை அழுத்தத்திற்கு உட்படும் போது அந்த இடைநிலை இழைகள் உடைந்துவிடுகின்றன. அவை புறஇரத்தத்தில் கலந்துவிடுகின்றன. இந்த சீரம்சைட்டோகெராட்டின் 18 எலிசா செயல்முறை மூலம் புறஇரத்தத்தில் அளவெடுக்கப்படும் போது அது ஒரு சிறந்த துளைத்தல் இல்லாத செயல்முறையாகப் பயன்படுகிறது.

தங்களிடமிருந்து ஊசியின் மூலம் 5 மி.லி. இரத்தம் எடுப்பதனால் எந்தவிதமான பக்க விளைவுகளும் ஏற்படாது என உறுதி அளிக்கின்றேன்.

தாங்கள் இந்த ஆராய்ச்சியில் பங்கேற்க நாங்கள் விரும்புகிறோம். இதில் பங்கு பெறுவதினால் நோயின் ஆய்வறிக்கையோ அல்லது சிகிச்சையோ பாதிப்புக்கு உள்ளாகாது என்பதையும் கூடுதல் செலவீனம் ஏற்படாது என்பதையும் தெரிவித்துக் கொள்கிறோம்.

முடிவுகளை அல்லது கருத்துக்களை வெளியிடும் போதோ அல்லது ஆராய்ச்சியின் போதோ தங்களது பெயர் மற்றும் அடையாளங்கள் வெளியிடப்படாது என்பதை தெரிவித்துக் கொள்கிறோம்.

இந்த ஆராய்ச்சியில் பங்கேற்பது தங்களின் விருப்பத்தின் பேரில் தான் இருக்கிறது. எந்நேரமும் இதிலிருந்து பின்வாங்கலாம் என்பதையும் தெரிவித்துக் கொள்கிறோம்.

இந்த ஆராய்ச்சியின் முடிவுகளை தங்களுக்கு அறிவிப்போம் என்பதை தெரிவித்துக் கொள்கிறோம்.

நீங்கள் இந்த ஆராய்ச்சியில் பங்குபெற விருப்பம் இருப்பின் இந்த தகவல்தாள் மற்றும் ஆராய்ச்சி ஒப்புதல் படிவத்திலும் கையொப்பம் இடுமாறு கேட்டுக் கொள்கிறோம்.

ஆராய்ச்சியாளர் கையொப்பம்

பங்கேற்பாளர் கையொப்பம்
/இடது கைவிரல் ரேகை

இடம் :
தேதி :

நோயாளியின் ஒப்புதல் படிவம்

ஆராய்ச்சி தலைப்பு:

"மதுபானம் சாரா கொழுப்பு கல்லீரல் நோயாளிகளைக் கண்டுபிடிப்பதில் சீரம்சைட்டோகெராட்டின் 18எம்30-ன் பங்கு பற்றிய ஆய்வு".

பங்கேற்பாளர் பெயர் : புற / உள் நோயாளி எண்:

வயது : பால் : கைபேசி/தொலைபேசி எண் :

முகவரி : ஆராய்ச்சி சேர்க்கை எண் :

.....ஆகிய நான் மரு. J.விநோதா, பட்டமேற்படிப்பு, மருத்துவ மாணவி, உயிர்வேதியியல் உயர்நிலைத் துறை, சென்னை மருத்துவக் கல்லூரி, சென்னை. மேற்கொள்ளும் ஆராய்ச்சியில் பங்கேற்க எந்தவித நிர்பந்தமின்றி, முழு சுதந்திரத்துடன் சுய நினைவுடன் முழு மனதுடன் சம்மதம் தெரிவிக்கின்றேன்.

இந்த ஆராய்ச்சி பற்றிய தகவல் தாளை பெற்றுக் கொண்டு அதன் முழு விவரங்களையும், நோக்கங்களையும் மருத்துவரின் மூலம் புரிந்துக்கொண்டு எனது மருத்துவ குறிப்புகள், பரிசோதனை முடிவுகள் மற்றும் இரத்தத்தை பயன்படுத்திக் கொள்ளவும் மேலும் இந்த ஆராய்ச்சியிலிருந்து எந்நேரமும் பின்வாங்கலாம் எனவும் அச்செயலினால் எந்த பாதிப்பும் ஏற்படாது என்பதையும் புரிந்துக் கொண்டு முழு மனதுடன் சம்மதம் தெரிவிக்கின்றேன்.

எனது பரிசோதனை முடிவுகளை அறிவியல் சார்ந்த அமைப்புகள் மற்றும் மருத்துவ இதழ்களில் வெளியிடுவதற்கு முழு மனதுடன் சம்மதிக்கின்றனே.

ஆராய்ச்சியாளர் கையொப்பம் பங்கேற்பாளர் கையொப்பம்
/இடது கைவிரல் ரேகை

இடம் :

தேதி :

PROFORMA

Date : Sample Id : _____

Name : Age : Sex : Ht (cm): Wt (kg) :

Waist circumference:

Pre/Post Menopausal:

Ethnicity: Community :

Duration of Symptoms/diseases :

Treatment history :

H/O alcohol intake in the last five years:

H/O jaundice

H/O previous surgeries

Other autoimmune diseases : _____ if any duration _____

Associated diseases with duration :

Type 2 Diabetes Mellitus □ Hypertension □ Ischemic heart disease □
Congenital liver diseases □

Hypothyrodism □ PCOD □ Hypercholesterolemia □

Gastric surgeries □ Inflammatory bowel disease □
Others □

Drug Intake:

Steroids □ Acetaminophen □ Tetracyclines □

Any other medications □ Smoking □ Passive smoking □

Alternative medicine intake □

Clinical History:

H/O upper abdominal pain _____

H/O nausea or vomiting _____

H/O loss of appetite _____

H/O recent jaundice _____

H/O breathlessness _____

H/o weight gain _____

H/O recent surgeries _____

O/E :

Pallor ____ Icterus _____ Lymphadenopathy _____

Pulse : B.P : Temp:

CVS/RS :

P/A :

Sample collection :

Date _____ Time _____

Sample analysis :

Date _____

Investigations:

Serum cytokeratin 18 M30 :

Serum fasting lipid profile :

Plasma Fasting blood glucose:

Serum liver function tests :

Serum uric acid :